MOLECULAR AND IONIC TRANSPORT ACROSS BIOLOGICAL MEMBRANES

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CONTENTS

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

The object of molecular and ionic transport studies in animal tissues is to explain fully such diverse physiological events as the secretion of acid by the gastric mucosa and the conduction of impulses in nerve. It appears from many sources that the solution of such problems lies within the cell membrane itself.

To some extent the penetration of molecules and ions through biological membranes can be explained by the operation of familiar physical forces; in other instances, evidently no quantitative description is at hand and the material transport appears to be 'metabolically-driven'. This latter kind of transfer was termed 'Active Transport' by August Krogh.

Before deciding whether or not a molecular species is actively transported by a membrane one needs an adequate theory of passive membrane behaviour. During the last few years an approach, based on the thermodynamics of irreversible processes (Prigonine, 1947; De Groot, 1951) has developed. This thermodynamic theory of flow in open systems defines formally the nature of the flows, indicates the important driving forces and derives quantitative relations between the flows and the forces. Moreover, this approach to biological transport phenomena promises success by expressing the thermodynamic flow parameters in terms of frictional coefficients open to kinetic interpretation.

1.2 THEORY OF PASSIVE TRANSPORT

The foundations of the thermodynamics of irreversible processes can be found in the texts of De Groot (1951) and Denbigh (1951). The applications of this approach to membrane transport have been outlined in the papers of Kedem and Katchalsky (1958, 1961, 1962, 1963), Katchalsky (1961), Kedem (1961), Dainty (1963), Dainty and Ginzburg (1963) and Ginzburg and Katchalsky (1963).

Provided that the system under consideration is near equilibrium it can be assumed that the fluxes are linear functions of all of the forces on the components of the system. If there are n flows $J_1 \ldots\ldots J_n$ and n corresponding forces $X_1 \ldots\ldots X_n$, then according to Onsager (1931) the following n equations can be written:

$$\begin{aligned} J_1 &= L_{11}X_1 + L_{12}X_2 + L_{13}X_3 + \cdots + L_{1n}X_n \\ J_2 &= L_{21}X_1 + L_{22}X_2 + L_{23}X_3 + \cdots + L_{2n}X_n \\ J_3 &= L_{31}X_1 + L_{32}X_2 + L_{33}X_3 + \cdots + L_{3n}X_n \\ &\vdots \\ J_n &= L_{n1}X_1 + L_{n2}X_2 + L_{n3}X_3 + \cdots + L_{nn}X_n \end{aligned} \qquad (1)$$

The parameters L_{ij} are called the Onsager coefficients; the diagonal L_{ii} are proportional to the classical coefficients such as thermal conductivity while the Onsager reciprocal relations state that

$$L_{ij} = L_{ji} \quad , \quad \text{for} \quad i \neq j$$

These 'cross-coefficients', $L_{ij}(i \neq j)$, express the possibility that the flow of any component i may be affected by the conjugate force X_j, of component j.

As will be shown later it is often advantageous to express the forces as linear functions

of the flows:

$$X_i = \sum_{j=1}^{n} R_{ij} J_j \qquad (2)$$

1.21 Permeation of Non-Electrolytes

Considering a homogeneous membrane separating an 'outer' solution O from an 'inner' solution I (both well-stirred) and denoting the electro-chemical potential of species i by $\bar{\mu}_i$ then the driving force per mole of substance flowing through a point x is

$$X_i = - \frac{d\bar{\mu}_i}{dx} \qquad (3)$$

where flow proceeds along the co-ordinate x perpendicular to the membrane surface. The force X_i gives the species i a velocity v_i and the rate of transport through unit area of membrane is

$$J_i = \frac{dn_i}{dt} = C_i v_i \qquad (4)$$

where n_i is the number of moles of species i and C_i is the local concentration (moles per unit membrane volume) of species i . This analysis finally gives:

$$J_i = \sum_j L_{ij} (\bar{\mu}_j^{O} - \bar{\mu}_j^{I}) \qquad (5)$$

In practice, convenience of measurement dictates the substitution of the electrochemical potential gradients by other forces. Such forces are the fall of electrical potential across the membrane, $\Delta V = V^{O} - V^{I}$, the gradient of osmolar concentration of species $\underline{i}$, $\Delta c = c^{O} - c^{I}$, and the net hydrostatic pressure, $\Delta p = p^{O} - p^{I}$, across the membrane. These forces are related to the electrochemical potential gradient by

$$\Delta\bar{\mu}_i = \bar{\mu}_i^{O} - \bar{\mu}_i^{I} = \bar{V}_i \Delta p + RT\Delta(\ln c_i) + e_i \Delta V \qquad (6)$$

where $\underline{\bar{V}_i}$ is the partial molar volume and $\underline{e_i}$ the electrical charge of species $\underline{i}$.

In the case of the transport of a non-electrolyte bicomponent aqueous solution, i.e. water (w) and a single solute (s), equation (6) takes the form:

$$\begin{aligned} \Delta\mu_w &= \bar{V}_w \Delta p - \Delta\pi / c_w \\ \Delta\mu_s &= \bar{V}_s \Delta p + \Delta\pi / c_s \end{aligned} \qquad (7)$$

where $\Delta\pi$ ($=RT\Delta c_s$) is the gradient of osmotic pressure across the membrane.

Denoting the flux conjugated to Δp by J_v and that conjugated to $\Delta\pi$ by J_D it can be shown that:

$$\begin{aligned} J_v &= \dot{n}_w \bar{V}_w + \dot{n}_s \bar{V}_s \\ J_D &= \dot{n}_s / c_s - \dot{n}_w / c_w \end{aligned} \qquad (8)$$

where $\dot{n}_i$ = no. of moles of species $\underline{i}$ passing through unit membrane area during unit time.

Hence the flow conjugated to Δp is the total volume flow, while the flow J_D is the relative flow of solute versus water, or exchange flow.

In all cases where dilute solutions are used the water flow ($\dot{n}_w \bar{V}_w$) can be identified with the volume flow J_v.

The phenomenological equations for J_v and J_D may be written

$$\begin{aligned} J_v &= L_p \Delta p + L_{pD} \Delta \pi \\ J_D &= L_{pD} \Delta p + L_D \Delta \pi \end{aligned} \tag{9}$$

Inspection of equation (9) clarifies the physical meaning of the three coefficients L_p, L_{pD} and L_D. The character of L_p is that of a pressure filtration coefficient or hydraulic conductivity as it represents the volume flow, J_v, per unit pressure difference Δp (when $\Delta \pi = 0$), whereas L_D defines the exchange flow when $\Delta p = 0$ and has the nature of a diffusion coefficient. The cross-coefficient, L_{pD}, defines the osmotic flow per unit osmotic gradient when $\Delta p = 0$ and also characterises an ultra-filtration rate produced by purely mechanical pressures. Staverman (1952) has shown that the maximum value for L_{pD} is given by an ideal semipermeable membrane fully preventing solute transport; in this case, $L_{pD} = -L_p = -L_D$ and Staverman called the ratio $(-L_{pD}/L_p)$ the reflection coefficient, σ, of the membrane. This parameter usually lies in the range $0 \leqslant \sigma \leqslant 1$.

Introducing σ into equation (9) gives:

$$J_v = L_p (\Delta p - \sigma \Delta \pi) \tag{10}$$

Previously physiologists have used the equation

$$J_v = L_p (\Delta p - \Delta \pi) \tag{11}$$

often called Starling's hypothesis and applied to studies of fluid flow across capillary walls. The general validity of this hypothesis was disproved when experiments were performed on membranes with large permeabilities to the solutes responsible for the osmotic pressure gradient, $\Delta\pi$, (cf. Durbin, 1960).

Equation (11) can be generalized to treat caseswhere non-penetrating solutes exist on one or on both sides of the membrane. Denoting the osmotic pressure of the impermeant solutes by $\Delta\pi_i$, then J_v is given by

$$J_v = L_p (\Delta p - \Delta\pi_i - \sigma \Delta\pi) \tag{12}$$

Besides σ another parameter is introduced when dealing with a single permeating non-electrolyte and water. Kedem and Katchalsky (1958) presented the following relationships:

$$i_s = (J_v + J_D)\bar{C}_s \tag{13}$$

and,

$$\omega = (L_p L_D - L_{pD}^2)\bar{C}_s / L_p \tag{14}$$

where $\bar{C}_s$ is the mean concentration of solute in the membrane. Equations (9) can then be transformed to

$$\begin{aligned} J_v &= L_p (\Delta p - \sigma \Delta\pi) \\ i_s &= \omega \Delta\pi + (1-\sigma)\bar{C}_s J_v \end{aligned} \tag{15}$$

where ω is related to the permeability coefficient, P_s, for the solute under conditions of zero volume flow, viz.

$$P_s = (\omega RT)_{J_v = 0} \tag{16}$$

The three coefficients L_p, σ and ω, appearing in the equation, (15), for solute and water flows, are a complete set of independent parameters necessary for a proper description of osmotic phenomena in the presence of permeating solutes.

1.22 Frictional Models

Spiegler (1958), MacKay and Meares (1959), Katchalsky (1961), Kedem and Katchalsky (1961), Dainty and Ginzburg (1963) and Ginzburg and Katchalsky (1963) have discussed membrane transport in terms of frictional models giving physical meaning to the parameters arising from irreversible thermodynamics. Dainty and Ginzburg (1963) envisaged a special 'lipid-pore' model of the cell membrane and obtained an explicit equation for σ involving the frictional coefficients for such a model. Assuming a predominantly lipid membrane with a few water-filled pores in which solute and solvent movements interact, Dainty and Ginzburg found the following formula for σ,

$$\sigma = 1 - \omega \bar{V}_s / L_p - K_s^c f_{sw}^c / (f_{sw}^c + f_{sm}^c) \qquad (17)$$

In this formula, K_s^c is the partition coefficient for the solute between the water in the pores and the external solutions; f_{sw}^c and f_{sm}^c are the frictional forces per mole of solute between solute and water in the pores and between solute in the pores and the pore wall when the solute is moving at 1 cm.sec^{-1} relative to the solid parts of the membrane and to the water in the pores.

This formula, (17), provides an easy test for the pore-model of cell membranes. Briefly, if there are water-filled pores in the membrane i.e. if there is any frictional interaction between the solute and water as they travel through the pores then

$$\sigma < 1 - \omega \bar{V}_s / L_p \qquad (18)$$

and measurements of σ, ω and L_p in a given system should decide the validity of the pore-membrane view.

Kedem and Katchalsky (1961) obtained an analogous formula for σ:

$$\sigma = 1 - \omega \bar{V}_s / L_p - K f_{sw} / \varphi_w^m (f_{sm} + f_{sw}) \qquad (19)$$

In this formula, K denotes the partition coefficient of the solute between the membrane and the external solutions, f_{sw} and f_{sm} the frictional coefficients in the membrane per mole of solute and φ_w^m is the volume fraction of water in the membrane. The model assumed here implies frictional drags between solute, water and the membrane phase. Kedem and Katchalsky considered that whenever equation (19) was found to be valid then this would be a good indication that solute flow occurs in a capillary system in the membrane.

1.23 Permeation of Electrolyte Solutions through Charged Membranes

In treating the case of permeation of a bicomponent aqueous solution of uni-univalent salt through a charged membrane, three flows must be considered -- that of the water J_o, of the cation J_1 and of the anion J_2 instead of two flows (J_s and J_v) occurring in the transport of non-electrolytes. Generally the three flows will be driven by three forces: the gradient of chemical potential of water ($-\frac{d\mu_o}{dx}$) and the gradients of electrochemical potentials ($-\frac{d\bar{\mu}_1}{dx}$) and ($-\frac{d\bar{\mu}_2}{dx}$) of the two ionic components. The phenomenological equations require six independent coefficients instead of the three Onsager coefficients sufficing to describe the transport of bi-component non-electrolyte solutions.

The situation simplifies, however, if no electrical current flows through the membrane since then $J_1 = J_2 = J_S$, where J_S is the flux of electrically neutral salt. Moreover, the definitions of σ, ω and L_p retain their validity and the use of

frictional models to find explicit formulae for σ and ω appears in the papers of Kedem and Katchalsky (1961, 1962). In particular these workers found that in the permeation of uni-univalent salt through an uncharged membrane the final equations for σ and ω were identical to those for non-electrolyte penetration (when $f_{1w} + f_{2w} = f_{Sw}$, and $f_{1m} + f_{2m} = f_{Sm}$). On the other hand, for a highly charged membrane, where the net density of the fixed membrane charges (mole/unit volume) greatly exceeds the concentration of salt in the membrane, the following formula for σ was found:

$$\sigma = 1 - \omega \bar{V}_s / L_p - K f_{sw} / \varphi_w^m (f_{2w} + f_{2m}) \qquad (20)$$

where $f_{Sw} = f_{1w} + f_{2w}$, and $\underline{K}$ is the partition coefficient of the salt between the membrane and the external solutions. There is an apparent identity between this equation and the corresponding equation (19) for non-electrolytes, but this similarity is confusing. In the case of non-electrolyte permeation the partition coefficient (occurring in the expressions for σ and ω) is only slightly dependent on concentration, whereas this parameter for an electrolyte solution and a charged membrane is usually proportional to the average salt concentration in the external solutions. In particular, σ in equation (20) is not a constant, and as ω increases with c_s (mean external salt concentration) the reflection coefficient decreases and may take negative values producing anamalous negative osmosis first described by Dutrochet in 1835.

1.3 THEORY OF ACTIVE TRANSPORT

Considerable difficulties have occurred in the definition of active material transfer across biological membranes. Ussing and Zerahn, however, developed criteria for the coupling of cellular metabolism and mass transport and experimentally demonstrated its existence (Ussing and Zerahn, 1951; Zerahn, 1956; Ussing, 1958). Recently Kedem (1961) has extended the formalism of irreversible thermodynamics to embrace the concept of active transport, and, although this approach is in its infancy, it has been included here because it is the soundest one at present.

1.31 Equations of Flow

Kedem (1961) considered a chemical reaction taking place within a membrane while the solvent and a number of solutes passed from one bathing solution into the other. For simplicity it was assumed that the reaction involved oxygen and that reaction flow, J_r, denoted the number of moles of oxygen consumed by unit area of membrane in unit time. F_r was the change of Gibbs free energy accompanying the conversion of one mole of the oxygen at constant temperature and pressure. It was supposed that all the flows were interdependent, including the chemical reaction; and the equations for the flows were written as follows:

$$J_i = -\frac{1}{R_{ii}}\left[\Delta\bar{\mu}_i + \sum_{\substack{j=0 \\ j\neq i}}^{n} R_{ij}J_j + R_{ir}J_r\right]$$

$$J_r = -\frac{1}{R_{rr}}\left[F_r + \sum_{j=0}^{n} R_{ri}J_i\right] \qquad (21)$$

with Onsager's relation:

$$R_{ij} = R_{ji} \qquad \text{and} \qquad R_{ir} = R_{ri}$$

Here $\Delta\bar{\mu}_i$ is the difference in the electrochemical potential of component $\underline{i}$ across the membrane and R_{ij} is a generalized resistance coefficient; $\underline{o}$ denotes the solvent. The reciprocal of the coefficient R_{ii} gives the permeability of the membrane to $\underline{i}$ provided all other flows are zero. The coefficient R_{ij} represents the interaction between flows J_i and J_j, and becomes negative when $\underline{i}$ and $\underline{j}$ 'drag' each other along. When $R_{ir} \neq 0$, then the flow of $\underline{i}$ is coupled to the reaction in the membrane.

Thus Kedem concluded that the resistance coefficients offered a phenomenological definition of active transport: "The transport of an ion is active if R_{ir} is different from zero..... With $R_{ir} = 0$, $\underline{i}$ may still be transported actively if $R_{ij} \neq 0$ and $R_{jr} \neq 0$. In this case the flow of $\underline{i}$ is not coupled directly to the reaction, but it is taken along with a 'carrier', that is itself transported by the reaction".

1.32 Criteria for Active Transport

Kedem (1961) on the basis of equations (21) proceeded to outline certain criteria for active transport across a membrane separating aqueous solutions of one uni-univalent salt (cation denoted by 1, anion by 2, solvent by $\underline{o}$) at the same hydrostatic pressure and temperature. As a practical example of the value of this thermodynamic description Kedem chose to examine the results of flow experiments on frog skin. Kedem ignored any frictional drag between the flows of cation and anion and assumed that there was no coupling between J_o and J_r. For the purpose of illustration I shall revert to the most general situation described by the following

equations:

$$
\begin{aligned}
J_o &= -\frac{1}{R_{oo}}\left[\Delta\mu_o + R_{o1}J_1 + R_{o2}J_2 + R_{or}J_r\right] \\
J_1 &= -\frac{1}{R_{11}}\left[\Delta\bar{\mu}_1 + R_{10}J_o + R_{12}J_2 + R_{1r}J_r\right] \\
J_2 &= -\frac{1}{R_{22}}\left[\Delta\bar{\mu}_2 + R_{20}J_o + R_{21}J_1 + R_{2r}J_r\right] \\
J_r &= -\frac{1}{R_{rr}}\left[F_r + R_{ro}J_o + R_{r1}J_1 + R_{r2}J_2\right]
\end{aligned}
\qquad (22)
$$

When frog skin is bathed on both sides by identical sodium chloride solutions (Ringer) there exist inwardly directed flows J_1, J_2 and J_o. The important question is whether these flows are 'active' or not.

To maintain identical electrochemical potentials at the skin surfaces a short-circuit current is drawn from the skin and the current density is given by

$$I = F(J_1 - J_2)$$

where F is the Faraday and I is the current density (Amp.cm^{-2}). It was found in frog skin that the flow of sodium ions was identical with the current and that J_o remained. This proves that $R_{2r} = 0$, $R_{12} = 0$ and $R_{20} = 0$. The conclusion must be that sodium ions are actively transported while chloride anions move passively. Equations (22) may now be reduced to the following:

$$
\begin{aligned}
J_o &= -\frac{1}{R_{oo}}\left[\Delta\mu_o + R_{o1}J_1 + R_{or}J_r\right] \\
J_1 &= -\frac{1}{R_{11}}\left[\Delta\bar{\mu}_1 + R_{10}J_o + R_{1r}J_r\right] \\
J_2 &= -\frac{1}{R_{22}}\left[\Delta\bar{\mu}_2\right] \\
J_r &= -\frac{1}{R_{rr}}\left[F_r + R_{ro}J_o + R_{r1}J_1\right]
\end{aligned}
\qquad (23)
$$

These equations suggest new experiments to determine the nature of J_o. For example, if the skin is bathed by an impermeant anion (sulphate), all net ion flow is stopped; if $J_o \neq 0$ in this condition then $R_{or} \neq 0$, and active water transport

exists in this tissue, whereas, if $J_o = 0$, it may still be possible that $R_{Io} \neq 0$, i.e. that there exists an electro-osmotic flow. This latter hypothesis can be tested by passing depolarizing currents across skins in sulphate Ringer. These experiments may settle the question of whether J_o is a direct active water transport or an electro-osmotic flow.

However, there is a kind of hidden difficulty in this approach. Frog skin and other epithelia possess at least two permeability barriers in series and the transport properties of these systems may produce subtle differences from the single-membrane system treated here (see Curran and McIntosh, 1962 and Kedem and Katchalsky, 1963).

In conclusion, attention is drawn to Kedem's considerations of the independent measurements necessary to characterize the active transport of ions. She particularly stresses the importance of short-circuit current, influence of ion flow on oxygen consumption and, finally, the membrane potential. Her conclusion is that the criteria developed from irreversible thermodynamics are less ambiguous than the flux ratio test of Ussing (1952).

1.4 TRANSPORT ACROSS EPITHELIA

Several epithelia, available as large flat sheets, are very suitable for the study of molecular and ionic transport; they often constitute systems where large material transfer occurs under conditions allowing access to the electrochemical potentials of the components of the bathing solutions. Moreover, the measurements of these fluxes and of oxygen consumption in such tissues is easily performed. Thus the parameters needed to distinguish between passive and active transport (Kedem, 1961) can be found simply and accurately. These advantages, however, are gained at the expense of the complicated circumstances of transport -- even in the simplest epithelium(one cell layer) the flux path includes at least two cell boundaries. Generally, intercellular flow is safely neglected because of the high diffusion and electrical resistances of these structures.

Evidence is mounting (Ussing et al., 1960 and Harris, 1956) that the transport mechanisms in epithelia do not differ, in principle, from those found in the single animal and plant cells. In nearly all epithelia, however, the cells responsible for net transport appear to have asymmetrical permeability characteristics. Besides this common feature another pattern emerges -- many epithelia have the ability to transport electrolyte solutions; the absorbate or secretion is isotonic in the kidney proximal tubule, intestine and gall bladder. Nevertheless, isotonicity is not universally found since the salt gland of birds produces a hypertonic secretion whereas the kidney tubules of fresh-water fish form a hypotonic one. The mechanism of ionic transport has attracted experimental interest for numerous reasons, but on an atomic level the number of water molecules transferred greatly exceeds the ion movements. Often the mechanism of water transport has been

ignored and there is no quantitative theory of the nature of the coupling, if any, between ionic and water transport resulting in a characteristic osmolarity for each epithelial absorbate or secretion. Previously there was a basic uncertainty in describing the nature of water movement since no thermodynamic theory of active transport and coupled flows was available. In this respect the theoretical approach of irreversible thermodynamics should be the remedy.

The following examples of net transport of ions and water across certain epithelia are reliably representative of a large literature on this topic.

1.41 The Amphibian Skin

The ability of isolated frog skin to maintain its inner (corium) surface at a positive electrical potential relative to its outer (epithelial) surface was discovered over a century ago by Du Bois-Reymond. Another important property of this tissue was first reported as early as 1892 by Reid; he demonstrated a small net transfer of water in the inward direction in the absence of hydrostatic or osmotic pressure differences. Huf (1935) observed an inward movement of chloride ions across isolated frog skin and Krogh (1937, 1938) found that the intact salt-depleted frog absorbed specifically sodium from salt solutions as dilute as 10^{-5} Molar. Subsequently, Koefoed-Johnsen and Ussing (1958) suggested that asymmetrical permeability played an essential role in the net transport of sodium chloride across the skin. They demonstrated the existence of an outer boundary permeable to sodium but impermeable to potassium and on inner boundary relatively permeable to potassium but impermeable to sodium. In their model a coupled potassium-sodium 'pump' on the inner barrier transferred potassium into the cell and sodium out of the cell; they showed that the active transport potential carrying chloride passively inwards was the sum of a potassium diffusion potential at the inner

surface and of a sodium diffusion potential at the outer one.

Studies on frog skin have produced two important quantitative relationships. Ussing and Zerahn (1951) found that when the bathing media were identical Ringers the total short-circuit current drawn from the skin was exactly equivalent to the active transport of sodium ions. Moreover, the rate of oxygen consumption of the skin, exceeding the 'resting metabolism' in the absence of sodium transport, was proportional to the rate of sodium transport; for each extra oxygen molecule consumed an additional movement of 18 sodium ions occurred (Zerahn, 1956; Leaf and Renshaw, 1957).

A puzzling problem emerging from recent work on frog skin is the relationship between active sodium transport and the non-osmotic water flux. Many workers envisaged that the water was being dragged inwards by sodium and (or) chloride ions. Recently, however, Kirschner et al. (1960) and Capraro and Marro (1963) have found evidence against a close relationship between net flows of sodium and water across the skin.

1.42 The Gastrointestinal Tract

Schematically the gastrointestinal system is viewed as a tube comprising in sequence: the stomach; the small intestine, including the duodenum, jejunum and ileum; and the large intestine or colon. In animals the stomach secretes hydrochloric acid which is neutralized in the intestine by sodium bicarbonate from the pancreas; sodium chloride and water are then reabsorbed by the intestine. There have been several reviews of acid production in the stomach (Heinz and Öbrink, 1954; Hogben, 1955) and of intestinal salt and water transport (Verzár and McDougall, 1936; Durbin et al., 1958).

Most vertebrates possess a gall bladder in which hepatic bile (produced by the

liver) is stored between meals before being discharged into the duodenum during digestion. Because of important recent work on the gall bladder it is discussed later under a separate heading.

The Stomach. The parietal, or oxyntic, cells of the gastric mucosa secrete acid in mammals at a strength of O.16Normal together with O.OlMolar potassium chloride. Both hydrogen and chloride ions are moved against an electrochemical gradient and Hogben (1951, 1952 and 1955) showed that the short-circuit current was equal to the net transfer of chloride ions exceeding that secreted as hydrochloric acid. Part of the chloride movement was an 'exchange diffusion' flux (see Ussing, 1952). Forte et al. (1963) concluded that the hydrogen and chloride 'pumps' in the isolated stomach of the frog were electrogenic i.e. capable of producing a net transport of electric charge, but unfortunately they did not study the bearing of this hypothesis, if any, on the net secretion of water into the lumen. Nevertheless, Durbin et al. (1956) found that the net water flow across the same tissue seemed to be independent of hydrogen ion secretion. Thus the driving force for this non-osmotic water flow remains undefined.

The Small Intestine. Curran and Solomon (1957) observed a linear relationship between net solute flow and water fluxes during absorption of NaCl from the rat ileum in vivo. They also found that when iso-osmotic mixtures of mannitol and NaCl were substituted for isotonic NaCl as the luminal fluid, the net water flux fell sharply, coincident with the decrease in net sodium flux. Thus they concluded that the water was moving passively following sodium and chloride ions out of the lumen. Across this tissue potential differences of only a few millivolts have been found and this is partly due to the large permeability to ions; but Curran and Solomon (1957) demonstrated that the active transport potentials of sodium and

chloride ions nearly neutralize one another. They concluded that the active transport of sodium and chloride ions across the intestine produced an osmotic driving force for water movement.

Curran (1960) studied the interrelationships between metabolism, sodium chloride transport and water absorption in an *in vitro* preparation of rat ileum; he found that, provided glucose was present in the mucosal solution, sodium and chloride ions were actively transported from mucosa to serosa while water absorption was passive and dependent on net solute transport.

The Large Intestine. The short-circuit current technique has been used to study ionic movements *in vitro* across the large intestine of the toad (Ussing and Anderson, 1955) and of the bullfrog (Cooperstein *et al.*, 1957). These tissues showed an active transport of sodium (mucosa to serosa); in the toad intestine the short-circuit current arose entirely from sodium flow, but in the bullfrog the net sodium flux was occasionally less than the short-circuit current suggesting the active transport of another ion.

Curran and Schwartz (1960) performed *in vivo* experiments on rat colon and found that sodium was actively transported (mucosa to serosa) while the chloride transport was passive. Performing experiments (similar to those of Curran and Solomon (1957) on rat intestine) they concluded that the water transport in the colon was purely passive and followed the NaCl out of the lumen osmotically.

1.43 The Urinary Bladder

Here, as in frog skin, sodium ions move preferentially in one direction (mucosa to serosa); Leaf (1955) demonstrated that toad urinary bladder maintained a potential difference (serosal surface positive) when bathed by identical Ringers and found that the short-circuit current and active sodium flux were equivalent.

Recently Frazier and Leaf (1963) have proposed that the sodium 'pump' is electrogenic. There is also a net flux of water similarly directed to sodium transport and both of these flows can be stimulated by hormones of the neurohypophysis (Ewer, 1952; Sawyer and Schisgall, 1956). However, no critical examination of the relationship between the flows of sodium and water across this tissue has been performed.

1.44 The Gall Bladder

Diamond (1962a, b, c) studied the transport of ions and water across the in vitro gall bladder of fresh-water fish. He found that this tissue concentrates bile by reabsorbing an isotonic salt solution and that the potential difference across this epithelium was exceedingly small. He considered that ionic movements (mucosa to serosa) were produced by the operation of a neutral sodium chloride 'pump'. Applying the criteria developed from the irreversible thermodynamics of membrane transport, he concluded that the net water flux was passive and coupled to the active salt movement. In discussing the kinetic mechanism of water transport across the gall-bladder Diamond felt that it was important that the ratio of fluid transported to solute transported was the same during passive diffusion of salt down its activity gradient and during active salt transport.

Recently Wheeler (1963) has studied similar problems in the wall of rabbit gall bladder in vitro; he found that the net water flux (mucosa to serosa) was directly proportional to the net solute transport (measured as sodium flux) and the transported solution was slightly hypertonic. He discovered that electrical potential differences were small (lumen positive) and flux ratio determinations indicated active transport of sodium and chloride but not potassium -- active anion and cation fluxes were not independent. Wheeler concluded that water movement

was dependent upon active salt transport but he refrained from offering a precise kinetic coupling mechanism.

Grim (1963) studied the mechanism of absorption of sodium chloride solutions from the canine gall bladder (in vivo), and he proposed that his observations were consistent with the active transport of (nearly) isotonic NaCl from lumen to blood by a 'solution pump' like pinocytosis occurring at a rate independent of luminal concentration. Such a model clearly requires further investigation since the specificity of sodium and chloride ion transport is difficult to explain. In his interesting paper Grim criticized the conclusion of many workers on epithelial water transport.

In particular, Grim pointed out that Diamond's (1962) estimation of the reflection coefficient, σ, for NaCl — an important parameter in the criteria for active versus passive water transport — presupposes that the water transport is passive. Assuming that re-absorption of water remained in Diamond's experiments when sucrose replaced NaCl in the lumen Grim obtained a lower value of σ (0.78) than Diamond's value (0.93). Diamond found that when sucrose replaced all NaCl in the lumen the gall bladder gained weight; he showed this was a passive property of the membrane since the gain in weight "persisted" in the presence of cyanide and iodoacetate. Grim's alternative hypothesis (i.e. σ = 0.78) predicts an increase in the weight gained after poisoning; this was apparently not observed and hence Diamond's contention that water movement is passive appears to be correct.

Moreover, Grim claimed that evidence for passive water transport across the rat ileum (Curran and Solomon, 1957) is inconclusive. He correctly stated that a linear relationship between solute and water transport, by itself, does

not decide which is moving passively, and suggested that the decrease in water flux occurring with the mannitol mixtures arose because of the changes in osmotic forces. His contention was that iso-osmotic mixtures of mannitol and NaCl may not exert the same effective osmotic pressure in the lumen as isotonic NaCl because of the different permeabilities of the gut to these solutes. This is a strong criticism and the whole question needs further investigation to vindicate the passive nature of water transport across this tissue. Grim also criticized Curran and Schwartz (1960) on the same grounds.

1.45 Summary

Table 1 outlines comparatively representative studies that have been made on net transport of ions and water across some epithelia. In the first three cases, cited in the table, the net water flux was measured under conditions of zero electrochemical potential difference. In the experiments on frog gastric mucosa and on toad urinary bladder no electrical short-circuiting was performed.

Strikingly this survey displays the present ignorance of the nature of water movement across epithelia and of its kinetic coupling to active ion flow (itself, a puzzling phenomenon).

TABLE 1

Tissue (in vitro)	Net Solute Flux μ.equiv.cm^{-2}hr^{-1}	Net Water Flux mg cm^{-2}hr^{-1}	No. of Water Molecules transported per Solute Atom transported	Proposed Mechanism of Water Transport	Reference
Frog skin	0.4 (sodium)	0.9	130	None	Capraro & Marro (1963)
Rat ileum	2.4* (sodium)	16*	370	Osmosis	Curran (1960)
	2.4* (chloride)				
Fresh-water fish gall bladder	2.0 (sodium)	15	420	Passively linked to active NaCl 'pump' (perhaps osmotically)	Diamond (1962)
	2.0 (chloride)				
Frog gastric mucosa	0.7 (hydrogen)	11	870		Durbin et al. (1956)
	3.8 (chloride)		(160)	None	Hogben (1955)
Toad urinary bladder	0.5 (sodium)				Leaf (1955)
		2	(220)	None	Matty & Green (1963)

* These figures have been calculated from the original fluxes expressed as μ.equiv./cm length of tissue.hr. Wood (1944) has estimated that 1 cm. length of rat ileum is equivalent to about 5 cm^2.

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2.2 METHODS

The experiments were performed on the skins of Rana temporaria at room temperature (16 - 20°C). Animals were killed by cutting the spine and pithing; abdominal skin was removed, cleaned of all adherences and washed in a volume of Ringer or sulphate Ringer. Table 1 gives the composition of the principal solutions used in this study. All of the salines were buffered with 'tris' at pH 7.6 - 7.7 and the use of these media will be indicated in the text or legends to Figures by the designated symbols. Salines A and B will be referred to as Ringer and sulphate Ringer respectively.

TABLE 1

COMPOSITION OF THE EXPERIMENTAL SOLUTIONS

Concentrations are given in millimoles per litre (mM/l.)

	Designation of solution								
	A	B	C	D	E	F	G	H	I
NaCl	97.5	-	97.5	97.5	97.5	-	-	-	-
$CaCl_2$	1	-	1	1	1	1	1	1	1
KCl	2.5	-	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Na_2SO_4	-	48.75	-	-	-	-	-	-	-
$CaSO_4$	-	1	-	-	-	-	-	-	-
K_2SO_4	-	1.25	-	-	-	-	-	-	-
Sucrose	-	-	50	100	150	50	150	250	350
'tris'	5	5	5	5	5	5	5	5	5

2.21 Measurement of Water Flow

Previous workers have used the skin-bag technique or volumetric apparatus to measure the net water transport across frog skin. Both methods have disadvantages In the first, simultaneous monitoring of potential difference (ΔV), short-circuit

current (I) and weight is exceedingly difficult; moreover, tissue weight is a possible variable parameter in this system. In volumetric technique rigorous temperature control and careful design of apparatus are required. Because of these difficulties a simple gravimetric apparatus, capable of measuring ΔV, I and net water flux (Fig. 1a), was constructed from perspex or teflon (Polytetrafluorethylene); net water flow across the skin was determined by changes in chamber weight after the fluid in compartment x had been sucked out through a glass tube (nozzle diameter 1.5 mm) by a 'Speedivac' vacuum pump, model 2SC2OA (Edwards High Vacuum Ltd., Sussex, England). A water trap was included in the suction line and the average fluid evacuation rate was 8 ml/sec.

The procedure for water flux measurement began by filling compartment y (volume 1.5 - 5 ml) with solution and placing the skin, corium upwards over the mouth of B; then ring A was tightly clamped to chamber B by four screws with nuts and washers. Any small pieces of skin protruding between A and B were removed and the whole chamber meticulously dried with Kleenex tissue. During subsequent handling of the chamber rubber gloves were worn. Volume x (1 ml) was filled with solution and this was renewed several times every 15 minutes throughout the entire experiment. After at least one hour of equilibration the fluid volume x was removed under a binocular dissecting microscope and the chamber was weighed on a Stanton Unimatic balance. The fluid volume x was replaced, then removed and the chamber reweighed. After six weighings were completed the weight of the chamber was expressed as a mean $\pm$ SD and the chamber was always weighed six times every two hours except in one series of experiments (see Table 3) where some observations were performed at hourly intervals. Evaporation from the skin surface produced no significant change in chamber-weight during any single weighing.

Fig. Ia. Apparatus for the measurement of net water flux across frog skin.

A and B – teflon chambers; exposed skin area = 1.25 cm^2.

1. – Ag–AgCl electrodes.

2. – short-circuit current electrodes.

3. – Ringer-agar medium (3% solution).

4. – Ringer-agar bridge.

5. – inlet tube .

6. – outlet tube connected to suction pump.

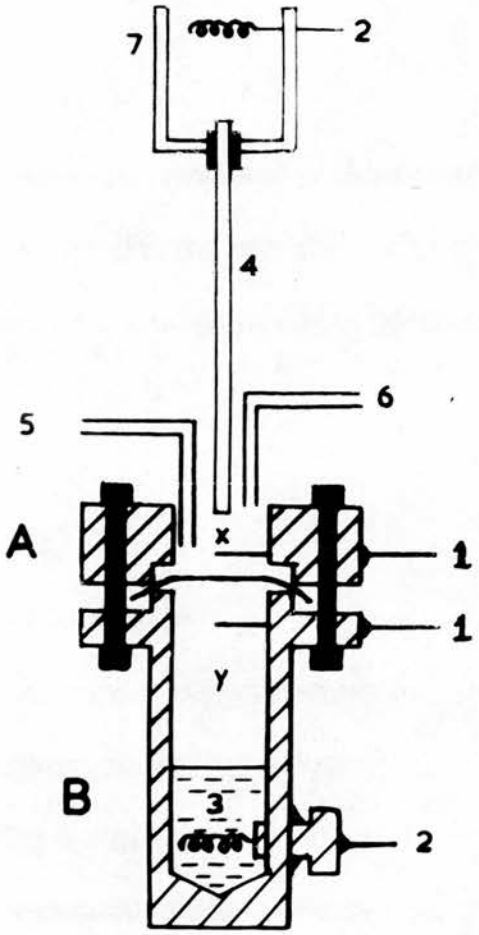
7. – perspex vessel containing Ringer.

Fig. Ib. Apparatus for the measurement of tissue-weight changes.

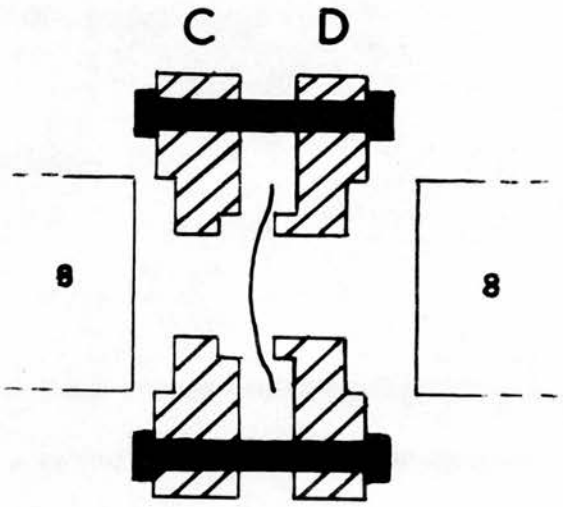
C and D – perspex rings; exposed skin area = 1.25 cm^2 .

8. – perspex chambers analogous to apparatus of Ussing and Zerahn (1951).

a.



b.



The exposed area of skin was 1.25 cm^2 and a typical SD of six weighings was ± 0.5 mg. This permitted a net water flux of 0.5 mg $cm^{-2}hr^{-1}$, over a two-hour period, to be recorded as significant (P = 0.01) by Student's *t* test. This value was chosen as the limit of significant measurement of water flow,

2.22 Measurement of Potential Difference and Short-Circuit Current

The potential difference was measured with Ag-AgCl electrodes (Fig. 1a) prepared immediately before experiments; these electrodes did not differ by more than 0.5 millivolt and were connected to a pH-meter No. 22, Radiometer, Copenhagen, by means of small metal plates with holes. Good electrical contact resulted when the connecting wires were under slight mechanical tension. These electrodes were 5 mm apart and no correction was applied for the potential drop in the solution during short-circuiting experiments.

In the perspex chambers the silver wires were sealed with Araldite; in the teflon chambers the holes for the potential electrodes were drilled undersize and a good seal obtained by forcing the wires into the chambers. Paraffin wax, applied warm, provided further external sealing when it solidified. The spiral current-electrode in chamber *B* was sealed into a teflon plug allowing easy installation.

Currents, passed across the skin, were measured with a Weston micro-ammeter. The mounting for the internal current-electrode was positioned by a Prior micromanipulator and the fluid volume *x* was renewed by inlet and outlet glass tubes during continuous short-circuiting of the skin.

2.23 Measurement of Tissue-Weight Changes

Changes in skin weight in various media were followed in an apparatus (Fig. 1b) employing the same drying procedure as in the gravimetric chamber. The

skin was clamped between the perspex rings C and D after it had been neatly cut to size (exposed area = 1.25 cm^2). This unit was fixed between two perspex chambers similar to the apparatus of Ussing and Zerahn (1951). Changes in tissue weight were determined by weighing the unit CD at two-hourly intervals as described in the water flux measurements. As there were two surfaces to be dried in these experiments the typical SD of six weighings was larger ($\pm$ 0.8 mg) producing a corresponding decrease in accuracy.

2.3 RESULTS

2.31 Control Experiments

Control experiments were performed to find if the change of chamber-weight could be attributed to the net water flow across the skin only, of if (1) leaking (2) water uptake by the chamber material or (3) tissue-weight change was the major component. As perspex can take up water this might alter the chamber-weight; teflon does not take up water and was a more reliable material on this count.

Paired pieces of skin from the same frog s were set up in the chambers; one skin from each pair served as a control (Ringer both sides) while the other piece was bathed in Ringer containing I mM/l. potassium cyanide (KCN). After a two-hour period of equilibration chamber-weight changes were measured over a further four hours. In a series of 32 two-hour periods involving 8 pairs the mean ± SE 'net water flux' was: for control skins an 'influx' of 1.2 ± O.3 mg $cm^{-2}hr^{-1}$, and for poisoned skins, a 'net flux' of O.O ± O.I mg $cm^{-2}hr^{-1}$. In a similar experiment with 4O two-hour periods involving I0 pairs, in which half were poisoned with 10^{-4} M/l. 2,4-dinitrophenol (DNP), the mean ± SE 'net water flux' was: for control skins an 'influx' of 1.4 ± O.2 mg $cm^{-2}hr^{-1}$, and for DNP-treated skins a 'net flux' of O.O ± O.I mg $cm^{-2}hr^{-1}$. During the control experiments a systematic decrease of the net water influx with time was observed and this was evident also in later experiments (Table 2) despite the maintenance of approximately steady electrical potentials. This observation disagrees with the work of Kirschner et al. (1960) who found a tendency for the flow to increase during experiments; these difficulties create the danger of bias in data collected in sequence from skins used in long experiments.

These control experiments strongly suggest that either (1) leaking or (2) water uptake by chamber material is not a major component of chamber-weight change; but it was also essential to know if there were any significant changes in skin weight in the flow experiments. After clamping pieces of skin in the apparatus (Fig. 1b) only the internal solution was stirred by aeration to simulate the experimental conditions of the water flow measurements. In a series of 19 two-hour periods involving skins from 11 animals there were only two significant weight changes in skins bathed in Ringer. MacRobbie and Ussing (1961) also found that the 'osmotic volume' i.e. the stratum germinativum of frog skin was surprisingly constant.

Tissue-weight experiments with poisoned skins were not performed because it seemed highly improbable that absence of 'net water flux' in these skins was caused by fortuitous cancellation of the components of chamber-weight change.

2.32 Osmosis

MacRobbie and Ussing (1961) made a careful study of the swelling and shrinking of the epithelial cells in frog skin in media of various tonicities and they obtained estimates of the osmotic permeabilities, or hydraulic conductivities, of the two epithelial membranes. They found that the outer membrane was relatively more impermeable to water than the inner membrane. It was decided to obtain independently a value for the hydraulic conductivity of the whole skin for comparison. Osmotic experiments were performed on paired pieces of skin from the same animals; one piece from each animal was poisoned by adding KCN to all experimental solutions (final concentration CN^-, 1 mM/l.). The skin pairs were subjected to the same conditions of osmotic pressure difference by bathing them with solutions C, D, E and Ringer, and each skin was exposed to one osmotic gradient only. Sucrose was considered to be completely impermeant, and an estimate of the hydraulic conductivity

of frog skin was found from a plot of net water flow against osmotic pressure difference across the skin (Fig. 2). In normal and poisoned skins the relationships between net water transport and the net osmotic driving force can be expressed mathematically:

Normal skins, $$J_v = (J_v)_o + L_p RT(C_s^i - C_s^o) \quad (1)$$

Poisoned skins, $$J_v = L_p RT(C_s^i - C_s^o) \quad (2)$$

where J_v is the net water influx ($cm^3 cm^{-2} sec^{-1}$); C_s^i and C_s^o the concentrations (mole cm^{-3}) of sucrose in the internal and external media; R, the gas constant; T, the absolute temperature; L_p, the hydraulic conductivity ($cm\ sec^{-1} atm^{-1}$) of frog skin and $(J_v)_o$ is the rate of fluid transport between identical Ringers in $cm^3 cm^{-2} sec^{-1}$.

The simplest description of Fig. 2 is that in normal and poisoned conditions the skin appears to have identical hydraulic conductivities i.e. $L_p = 3.9 \times 10^{-7}$ cm sec^{-1} atm^{-1}. This value agrees roughly with MacRobbie and Ussing's estimate of 10^{-7} as the upper limit cm $sec^{-1} atm^{-1}$ (in sulphate Ringer) (for the outer membrane which is the rate-limiting barrier to osmotic water flow. It is also interesting that the non-osmotic water influx, $(J_v)_o$ appears to be unaltered by the different osmotic pressures on the surfaces of the skin.

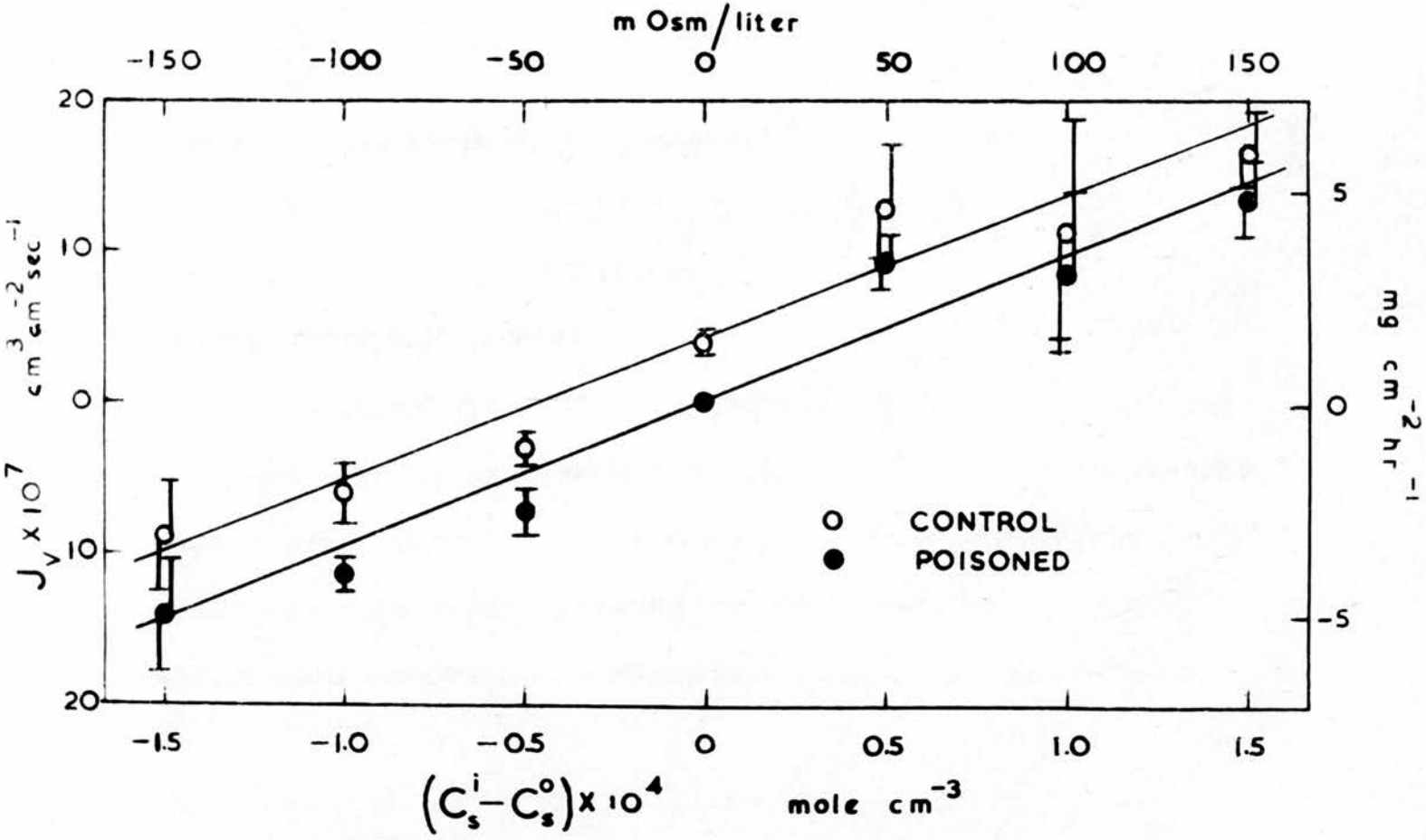
Recently Kedem and Katchalsky (1963) have shown theoretically that non-linear rate laws may hold for composite membranes (analogous to frog skin) and in view of this and of the effects of sucrose on the electrical resistance of the skin (Ussing and Andersen, 1955) it is surprising that such a high linear correlation for osmosis has been found. Coefficients of correlation of the regression lines for control and poisoned skins were $r = 0.9261$ and $r = 0.9425$.

Fig. 2. Net water flow as a function of osmotic gradient across the skin. Each point represents the average value of 10 measurements obtained in 5 experiments using a pair of skins from the same frog for each experiment, one serving as control and one treated with 1 mM/l. KCN. The bars indicate $\pm$ SD. Non-zero values of $(C_s^i - C_s^o)$ were produced by bathing one surface of the skin with Ringer and the other surface with C, D or E. Regression lines were found for control and poisoned skins:

Controls: $J_v = 4.63 \times 10^{-7} + 9.70 \times 10^{-3} (C_s^i - C_s^o)$

Poisoned: $J_v = 9.29 \times 10^{-9} + 9.84 \times 10^{-3} (C_s^i - C_s^o)$

The slope of these lines is given by $(L_p RT)$ and hence L_p was determined.


m Osm / liter
-150
-100
-50
0
50
100
150
20
10
0
10
20
5
0
-5
$J_v \times 10^7$ cm^3 cm^{-2} sec^{-1}
mg cm^{-2} hr^{-1}
CONTROL
POISONED
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
$(C_s^i - C_s^o) \times 10^4$ mole cm^{-3}

2.33 Effect of 'Pitressin' on Non-Osmotic Flow

There have been numerous recent studies of the effect of anti-diuretic hormone (ADH) on frog skin (Koefoed-Johnsen and Ussing, 1953; MacRobbie and Ussing, 1961; Whittembury, 1962, and Herrera and Curran, 1963). Experiments have demonstrated that ADH increases the hydraulic conductivity of the skin, while leaving the diffusion permeability to water nearly unaltered. Moreover, ADH is known to increase the rate of active sodium transport across the skin (Fuhrman and Ussing, 1951, and Ussing and Zerahn, 1951). However, no investigation has been reported on the effect of ADH on non-osmotic flow across the skin and such experiments may help to decide whether or not water and active sodium flows are linked during stimulation.

In all of the following experiments the tissue was equilibrated with the media for at least one hour before commencing the two-hourly weight measurements. ADH ('Pitressin', Parke, Davis & Co. Ltd.) was always added to the internal solution to give a final concentration of 0.1 U/ml after an initial two-hour period of control measurement had elapsed.

Fig. 3 shows a typical result of experiments on the effect of ADH on non-osmotic flow and short-circuit current ($\underline{I}$) across the skin. Strikingly there is a transient increase in the water transport while there exists a sustained increase in active sodium flux.

This transient is also evident in the presence of osmotic flows across the skins after the addition of ADH (Fig. 4). The broken line represents the calculated regression line for the control period; the solid lines, A and B, represent the calculated regression lines for the first and second two-hour periods after ADH is added.

A possible explanation of this transient phenomenon is that the impurity level of Oxytocin in ADH is causing a tissue contraction i.e. fluid is passing from the skin

Fig. 3 The effect of ADH on short-circuit current ($\underline{I}$) and non-osmotic flow across frog skin. ADH was added at a time indicated by the arrow.

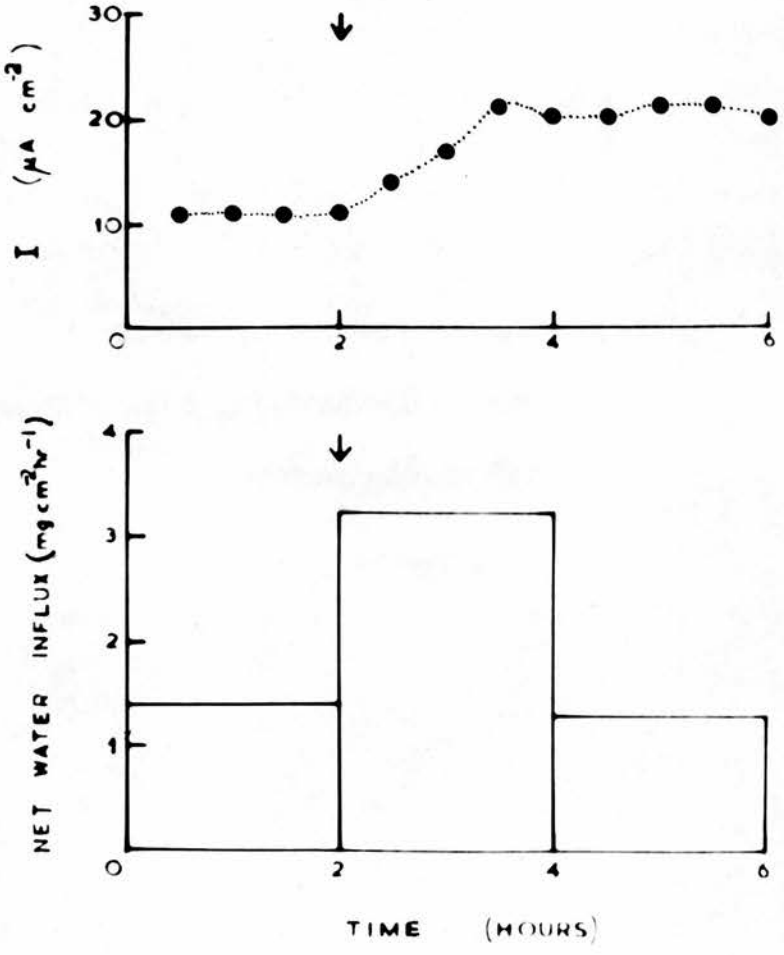
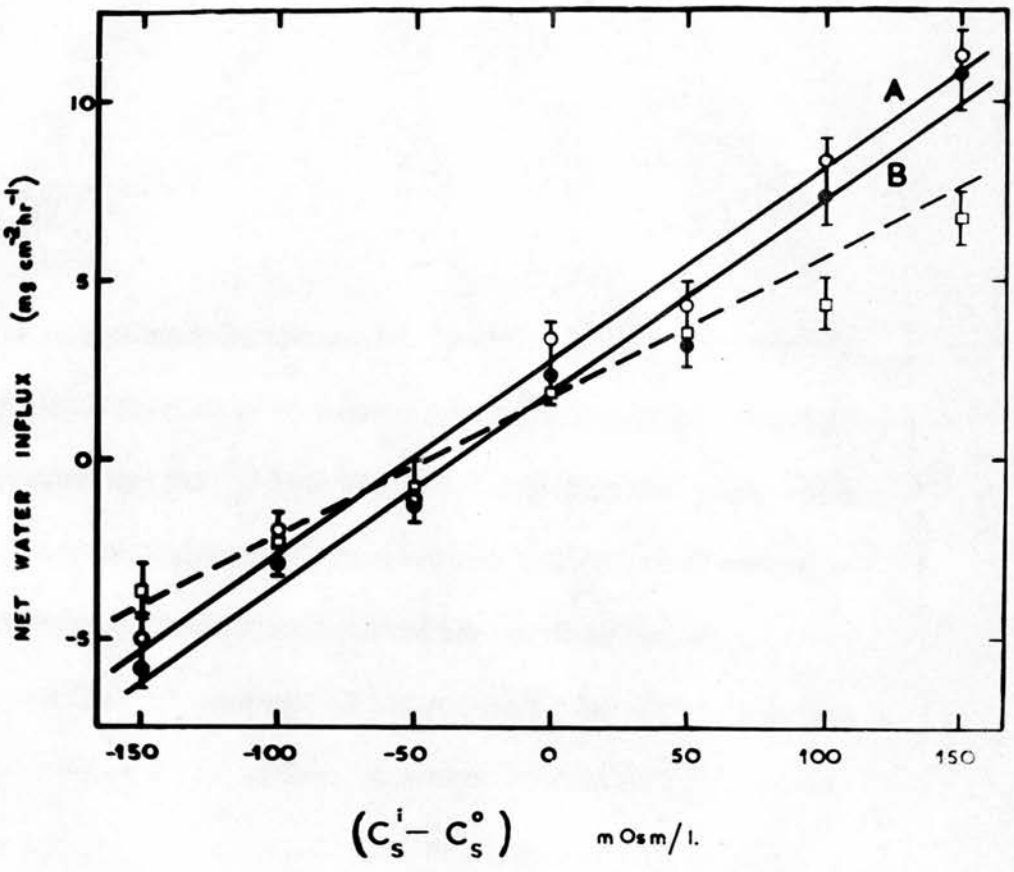

I (μA cm⁻²)
30
20
10
0
2
4
6
NET WATER INFLUX (mg cm⁻² hr⁻¹)
4
3
2
1
0
2
4
6
TIME (HOURS)

Fig. 4. Net water flux as a function of the osmotic gradient across the skin. Each point represents the average value of 10 measurements on 5 skins. The bars indicate ± SE. C_s^i and C_s^o are the concentrations of sucrose in the internal and external Ringer solutions. Each skin served as its own control; the broken line represents the control period while the solid lines, A and B, represent the first and second two-hour periods after treatment with ADH. □, control period; ○ and ●, first and second period.


NET WATER INFLUX (mg cm⁻² hr⁻¹)
10
5
0
-5
A
B
-150
-100
-50
0
50
100
150
(C_s^i − C_s^o)
mOsm/l.

into the internal solution. Exactly analogous tissue-weight experiments on six skins gave a mean ± SE decrease in weight: for the first two-hour period (after addition of ADH), 0.7 ± 0.3 mg, and for the second two-hour period, 0.5 ± 0.2 mg. At worst these changes would produce 'net influxes' of 0.3 and 0.2 mg $cm^{-2}hr^{-1}$. Therefore, the transient appears to be a genuine increase in non-osmotic flow; other supporting evidence is described in 2.37.

Stimulation of active sodium transport in frog skin by treatment with atropine sulphate produced a corresponding increase in the non-osmotic flux (Kirschner et al., 1960) and this seems to suggest a coupling of the flows. However, in the experiments reported here ADH produced no sustained increase in non-osmotic flow. Herrera and Curran (1963) reported that ADH creates a transient increase in sodium transport in late Spring whereas during Winter a permanent increase is obtained. Since my experiments were performed during Winter months they agree well with these latter observations and seem to suggest not that sodium and water flows are necessarily linked (for example, by electro-osmosis) but that ADH affects a rate-controlling site of the non-osmotic flow. Perhaps this site is the outer membrane. That the non-osmotic flow is altered by changes in the hydraulic conductivity of the outer membrane, may mean that there are local differences of osmotic pressure across this membrane and that these are the driving forces for this flux. Any explanation of the transient nature of the increase in water flux is complicated by the changes in cellular ionic contents possibly following the addition of the hormone and by the effects that these changes might have on the permeability barriers of the skin.

2.34 Determination of σ_{NaCl}

Diamond (1962) developed several criteria for deciding whether water transport in the gall bladder was active or passive. His theory rested on three passive

properties of the membrane: L_p, the hydraulic conductivity; σ_{NaCl}, the reflection coefficient for NaCl in the membrane and ω_{NaCl} where (ω_{NaCl}.RT) is the NaCl permeability when there is no volume flow. Staverman (1951) has shown that the osmotic pressure exerted at a membrane by a solution containing a diffusible solute is less than the theoretical value; σ for a given solute and membrane is the ratio of the observed osmotic pressure to the theoretical van't Hoff value.

To measure σ_{NaCl}, a large piece of skin from an animal was cut into four sheet of suitable size for the gravimetric chambers. Each skin had a different external medium (F, G, H or I) while the internal medium was Ringer. In this condition there could be no active sodium transport and the net water flow across each skin was measured over four two-hour periods. Regression lines were fitted to these data and the concentration (C_s^o) of sucrose, when no net water transport occurred, was determined. As sucrose may be considered effectively impermeant, σ_{NaCl} was calculated from $C_s^o = \sigma_{NaCl} C_{NaCl}$, where C_{NaCl} is the osmolarity of NaCl in Ringer. In two experiments regression lines with coefficients of correlation, $r = 0.9720$ and $r = 0.9791$, gave values for C_s^o of 196 ± 26 and 194 ± 22 mOsm/l. (In both cases, $\pm$ standard error of estimate). Since $C_{NaCl} = 181$ mOsm/l., σ_{NaCl} was found to lie within 0.94 - 1.23 and within 0.95 - 1.19. This parameter was similarly measured for skins poisoned with 1 mM/l. KCN and in two experiments regression lines ($r = 0.9670$ and $r = 0.9580$) gave σ_{NaCl} within 0.79 - 1.11 and within 0.90 - 1.25. The precision of these determinations was not high enough to offer a reliable value for σ_{NaCl} other than $0.8 < \sigma_{NaCl} < 1$.

2.35 Relationship between Sodium Transport and Non-Osmotic Flow

Capraro and Garampi (1956) suggested that the net water influx across frog skin was electro-osmotic in character i.e. that the net sodium transport provided the

driving force for the water movement. From data, already obtained independently on net transport of sodium and water, the ratio of water molecules (allegedly) dragged per sodium ion lies in the range 50 - 300 and it suggested that it might be promising to measure $\underline{I}$ and net water flow across completely short-circuited skins. Fig. 5 shows the results of such experiments on skins from 22 animals. There appears to be no good correlation between sodium and water influxes (coefficient of correlation, $r = 0.6651$).

Several workers (Kirschner et al., 1960 and Capraro and Marro, 1963) have found no correlation between $\underline{I}$ and net water movement during inhibition of the sodium 'pump' by eserine and DNP. Fig. 6 shows the effects on $\underline{I}$ and net water influx when the skin is treated with Ouabain (10^{-2} mM/l.). Ostensibly water influx remains while active sodium transport decays, but in four analogous tissue weight experiments with Ouabain there was an average loss of 5.5 mg during the first two-hour period while no effective change occurred during the second two-hour period. These changes of tissue-weight make the interpretation of this experiment complex.

2.36 Replacement of External Sodium by Choline

Kirschner et al. (1960) discovered the existence of net water influx (0.8 mg $cm^{-2}hr^{-1}$) in conditions of no active sodium transport i.e. external sodium was replaced by choline. These results might be explained by a constant tissue swelling and, therefore, the experiments with external choline Ringer were repeated. Choline Ringers were prepared immediately before use and the concentrations of all constituents except sodium were the same; Kirschner et al. (1960) have shown that such choline Ringers are isotonic. Table 2 shows that a net inward movement of water was observed and that this flow was smaller than that of the controls. In Kirschner's experiments a constant entry of water into the skin from the external

Fig. 5. Net water influx across completely short-circuited skins as a function of the short-circuit current. As the water flow measurements were taken over two-hour periods, average values of short-circuit current were calculated (from plots of $\underline{I}$ against time) in the few cases where this parameter was varying significantly.

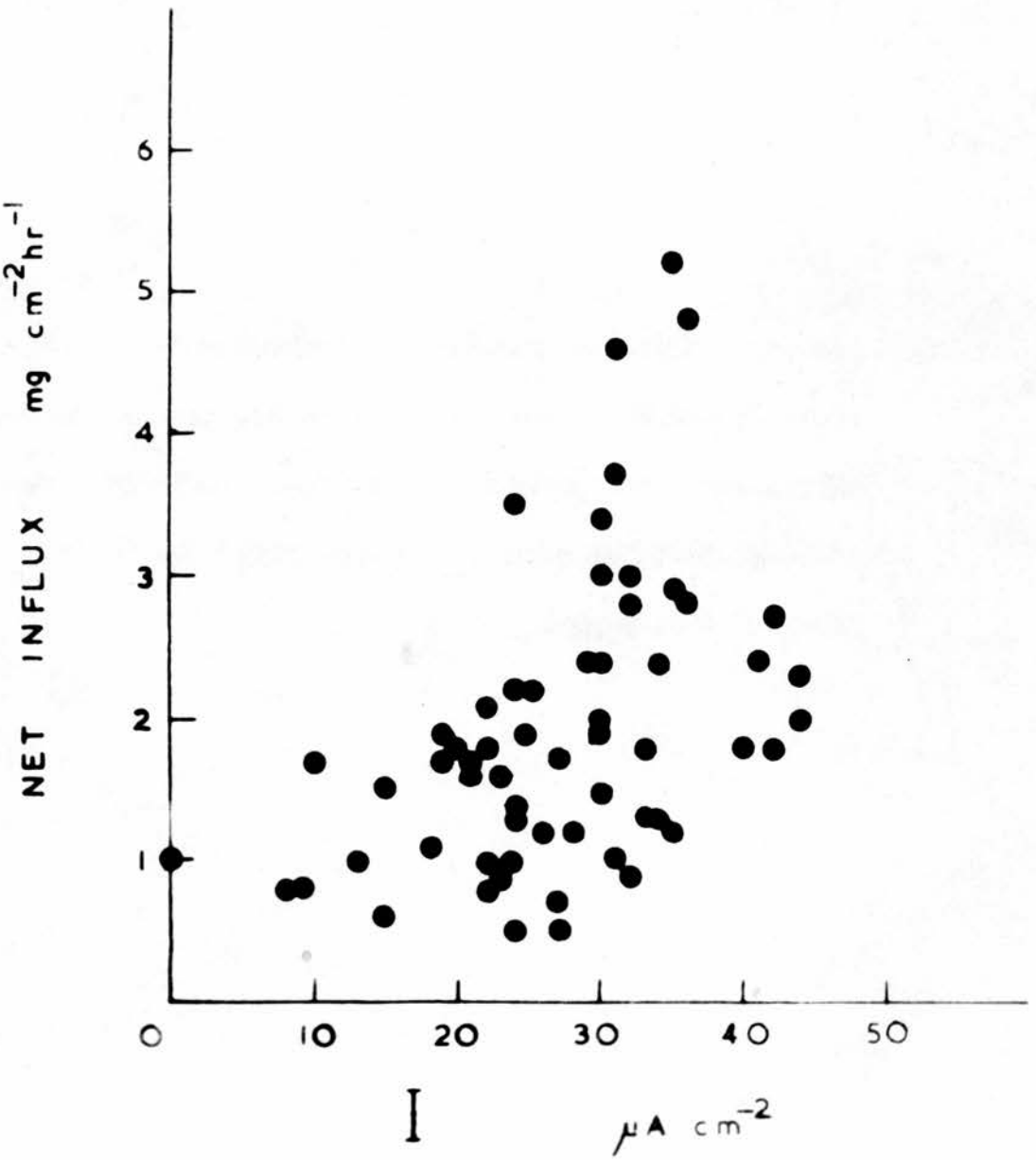
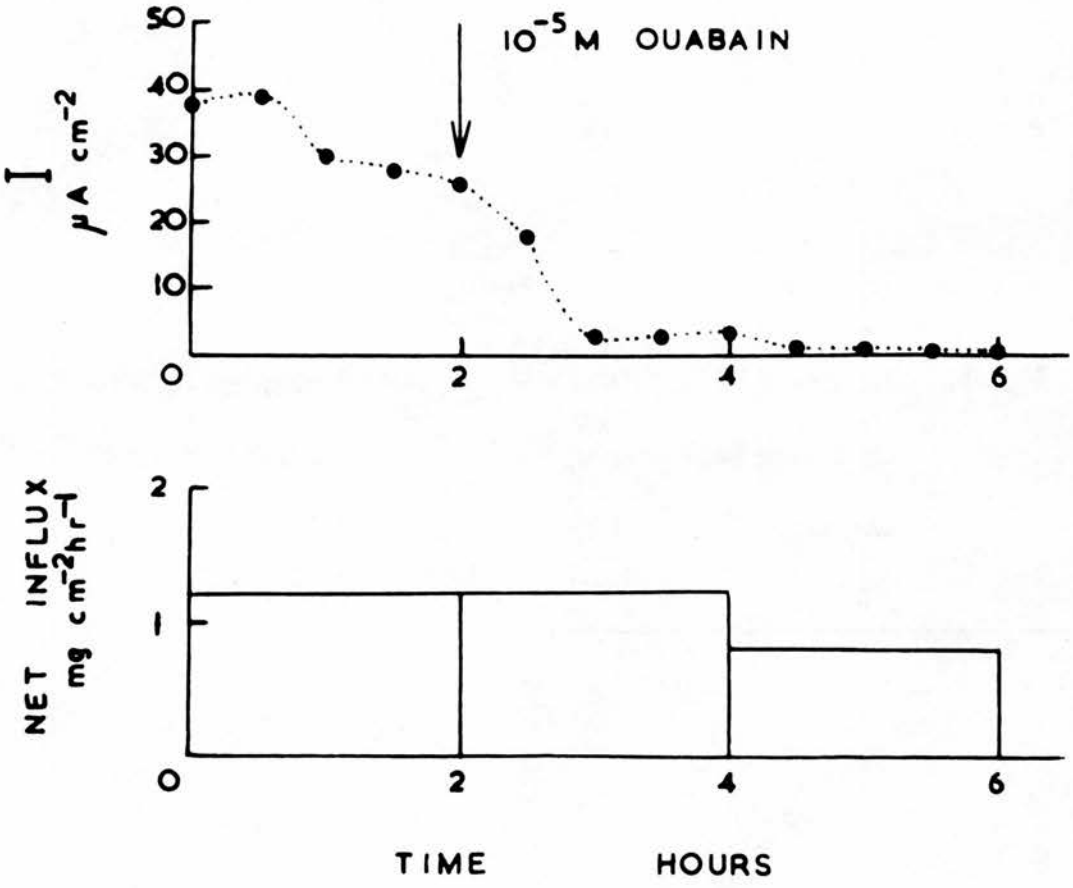

NET INFLUX mg cm^{-2}hr^{-1}
6
5
4
3
2
1
0
10
20
30
40
50
I
μA cm^{-2}

Fig. 6. The effect of Ouabain on the $\underline{I}$ and net water influx when it is applied to the inside surface of the skin at a time indicated by the arrow.


50
40
30
20
10
0
I
μA cm⁻²
10⁻⁵ M OUABAIN
2
4
6
NET INFLUX
mg cm⁻² hr⁻¹
2
1
0
2
4
6
TIME
HOURS

medium was necessary to explain this phenomenon whereas in my system that proposal would produce no net change in chamber-weight. It seems safe to consider that this water influx is genuine.

TABLE 2

WATER INFLUX ACROSS PAIRED FROG SKINS

Animal	External Solution	Net water influx (mg $cm^{-2}hr^{-1}$)		
		0 - 2 hrs.	2 - 4 hrs.	4 - 6 hrs.
1	Choline Ringer	2.9	1.8	1.4
	Ringer	5.8	3.0	2.0
2	Choline Ringer	0.0	0.0	0.0
	Ringer	1.4	1.3	1.4
3	Choline Ringer	1.9	1.0	0.0
	Ringer	2.0	1.6	0.9
4	Choline Ringer	0.0	0.0	0.0
	Ringer	1.6	1.0	0.8
5	Choline Ringer	0.0	0.0	0.6
	Ringer	1.8	1.9	1.3
6	Choline Ringer	0.8	0.6	0.6
	Ringer	0.0	0.0	0.0
	Choline Ringer		Mean $\pm$ SE = 0.6 $\pm$ 0.2	
	Ringer		Mean $\pm$ SE = 1.5 $\pm$ 0.3	

Internal solution in all experiments was Ringer. All net fluxes of water less than 0.5 mg $cm^{-2}hr^{-1}$ have been called zero.

However, this flux may arise from a difference in the osmolalities of the bathing media; this explanation implies an osmotic coefficient for choline chloride of about 0.8 which would have been detected easily in Kirschner's checks (by freezing-point depressions) of the total solute concentration in his choline Ringer, and therefore it is considered that this water flux is non-osmotic.

2.37 Experiments in Sulphate Ringer

Since the sulphate anion may be considered practically impermeant in frog skin, there can be no net transport of sodium ions across skins in sulphate Ringer. Therefore, a study of net water flux across skins in this medium might provide valuable evidence about the linkage, if any, of the two flows. Fig. 7 shows the net water fluxes across 25 skins in sulphate Ringer; evidently there is no net water transport when net ionic movement is absent. This situation provides adequate circumstances for testing the effect of passing depolarizing currents across the skin. Table 3 gives the results of current passage — presumably sodium ions — from external to internal medium. These results suggest an electro-osmotic drag of about 40 water molecules by every sodium ion. An alternative explanation is that the passage of these electrical currents causes a tissue shrinkage i.e. a loss of water to the internal medium. In five tissue weight experiments, designed to test this hypothesis, there was no such water loss when currents of 100 μA cm^{-2} were passed over one-hour periods.

Since skins in sulphate Ringer showed no non-osmotic flow, the effect of ADH on 8 skins bathed in sulphate Ringer was studied to discover whether or not the hormone would produce a 'net water influx'. The mean $\pm$ SE 'net water influx' was: for the first two-hour period (after ADH addition), 0.2 ± 0.2 mg $cm^{-2}hr^{-1}$, and for the second two-hour period, 0.1 ± 0.1 mg $cm^{-2}hr^{-1}$. These observations seem to be consistent with the results and conclusions in 2.33. Absence of non-osmotic flow in sulphate Ringer may follow from the fact that the 'non-osmotic' driving force arises partially from the presence of NaCl in the external Ringer i.e. the permeation of NaCl through the outer boundary may create an effective osmotic pressure across this membrane (see Chapter Three). Thus any increase in the hydraulic conductivity

Fig. 7. Net water movement across skins bathed on both sides by Sulphate Ringer.

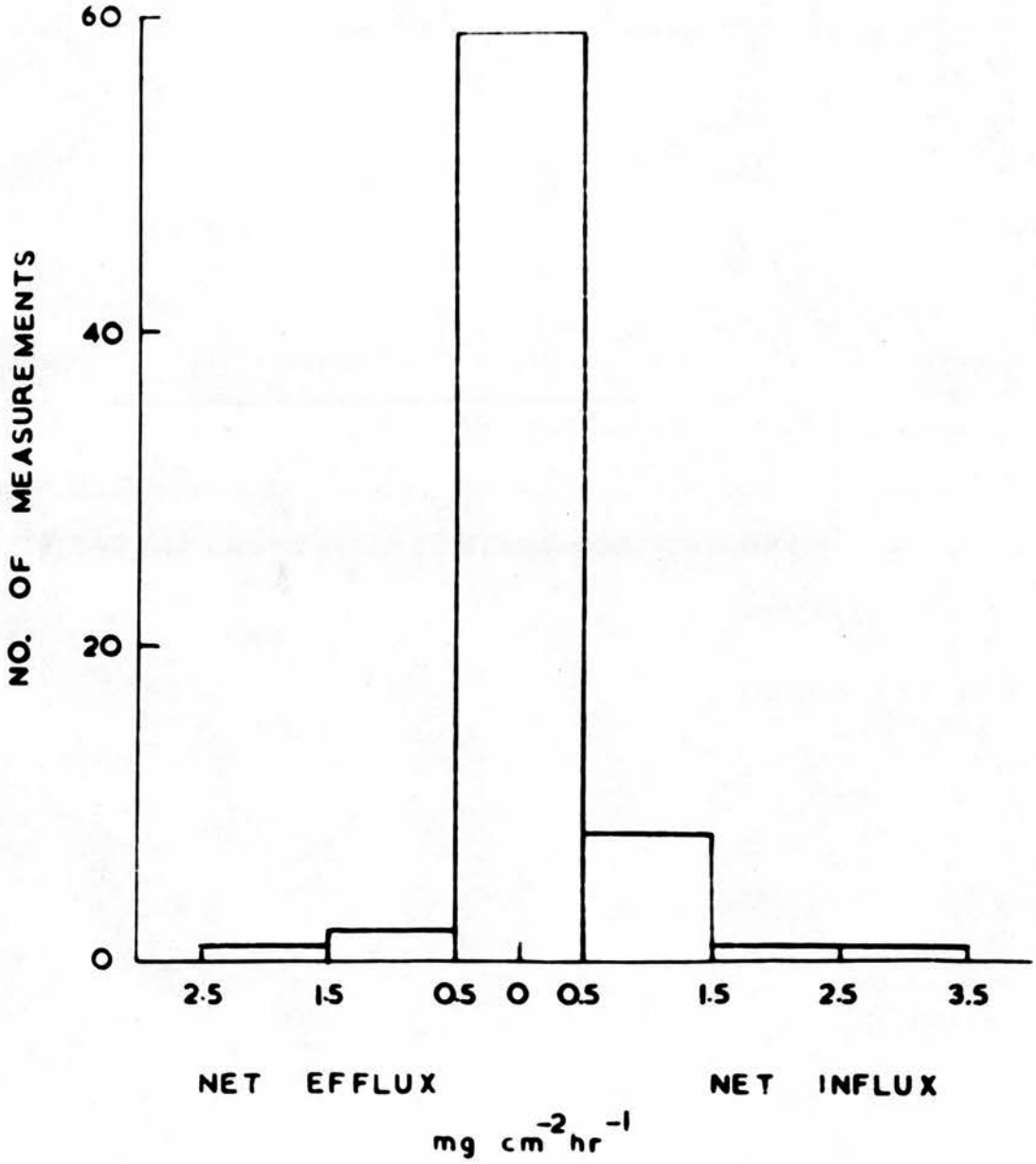

60
40
20
0
NO. OF MEASUREMENTS
2.5
1.5
0.5
0
0.5
1.5
2.5
3.5
NET EFFLUX
NET INFLUX
mg cm-2 hr-1

TABLE 3

EFFECT OF ELECTRICAL CURRENTS ON WATER TRANSPORT

	Net water influx (mg $cm^{-2}hr^{-1}$)		
Skin	0 - 2 hrs.	2 - 4 hrs**	4 - 6 hrs.
1	0.0	0.0	0.0
2	1.1	1.6	0.8
3	0.0	1.3	0.0
4	0.0	1.8	0.0
5	0.0	2.2	0.0
16	0.0	2.0	0.0
17	0.0	1.8	0.0
18	0.0	1.3	0.0
19	0.0	1.9	0.0
20	0.0	0.6	0.0
Mean ± SE		1.4 ± 0.2	
	0 - 2 hrs.	2 - 3 hrs.*	3 - 5 hrs.
6	0.0	2.6	0.0
7	0.0	3.5	0.0
8	0.0	2.9	0.0
9	0.0	3.4	0.0
10	0.0	4.1	0.0
11	0.0	2.6	0.0
12	0.6	2.4	0.0
13	0.0	1.9	0.0
14	0.0	2.0	0.0
15	0.0	2.5	0.0
Mean ± SE		2.8 ± 0.2	

Skins were bathed on both sides by sulphate Ringer.

** During this period a current density of 50 μA cm^{-2} was passed. It is assumed that this current was carried by a net inward movement of sodium ions.

* A current density of 100 μA cm^{-2} was passed during this hour.

All net fluxes of water less than 0.5 mg $cm^{-2}hr^{-1}$ have been called zero.

of this membrane in sulphate Ringer would not produce a non-osmotic flow because no osmotic driving force is present.

A study of osmotic flow across frog skin in sulphate Ringer gave a hydraulic conductivity, L_p, of about 1.5×10^{-7} cm $sec^{-1}atm^{-1}$ significantly lower than L_p in

Ringer. This was not a seasonal effect and I have no adequate explanation for this discrepancy.

2.4 DISCUSSION

There are several mechanisms which might produce a net inward transport of water across frog skin, and briefly these hypotheses may be discussed under the headings: pressure-driven flow, classical osmosis, 'co-diffusion', thermo-osmosis and electro-osmosis.

2.41 Pressure-Driven Flow and Classical Osmosis

It might be suggested that the non-osmotic water flow was pressure-driven, but calculation shows this hypothesis to be absurd. From the osmotic experiments on frog skin an estimate of the hydraulic conductivity was obtained, and, hence, it is possible to estimate the pressure difference required to produce the observed water influx. Taking L_p for frog skin as 4×10^{-7} cm $sec^{-1} atm^{-1}$ and the non-osmotic flow of water as 2.8×10^{-7} $cm^3 cm^{-2} sec^{-1}$, then the pressure difference required to produce this flow is 0.7atm.

Another contention might be that, if the net transport of NaCl across the skin increased the osmolarity of the inner solution relative to the outer medium, then water might follow salt inwards osmotically. Again it is possible to conclude by calculation that this hypothesis is unlikely to be correct. From the L_p and net water influx values it was calculated that the net osmotic pressure difference necessary to produce the flow is approximately 25 mOsm/l. It is conceivable that local differences in osmotic pressure of this magnitude may exist across such a complex structure as frog skin, but more experimental evidence is necessary before this hypothesis can be accepted. The possibility of the existence of an osmotic pressure difference across the outer membrane has been discussed in 2.33 and 2.37.

2.42 Co-Diffusion

Diamond (1962) concluded that net water transport in fish gall bladder was linked to the active NaCl flux by a process analogous to passive 'co-diffusion' of salt and water. He calculated expressions for the osmolarity of the absorbate solution on the basis of two different assumptions about the nature of the water movement. Assuming an active water 'pump' and passive solute movement, the osmolarity of the transported solution was:

$$\frac{M_s}{J_v} = \frac{C_s L_p (1-\sigma_s) - C_s \bar{V}_s \omega_s}{L_p}$$

where M_s is the flux of solute s in moles $cm^{-2}sec^{-1}$ and $\bar{V}_s$ is the partial molar volume of the solute (ml/mole). All other terms have been described before.

The other case considered was the linkage of passive water transport to an active solute 'pump' and the corresponding expression was:

$$\frac{M_s}{J_v} = \frac{\omega_s + C_s L_p (1-\sigma_s)^2}{L_p (1-\sigma_s)}$$

The experimental values of these parameters for frog skin are:

$L_p = 4 \times 10^{-7}$ cm $sec^{-1} atm^{-1}$

$0.8 < \sigma_{NaCl} < 1$

$\omega_{NaCl} = 8 \times 10^{-12}$ mole $cm^{-2} sec^{-1} atm^{-1}$

$C_{NaCl} = 1.8 \times 10^{-4}$ mole cm^{-3}

$\bar{V}_{NaCl} = 22$ cm^3 $mole^{-1}$

ω_{NaCl} is calculated from (P_{NaCl}/RT) where P_{NaCl} is taken as 2×10^{-7} cm sec^{-1}; this estimate is based on Morel's (1958) value for the sodium permeabilityof the inner membrane. Unfortunately no measurements have been made of P_{NaCl} under conditions of zero volume flow.

Active transport of water: $-0.08 < M_s/J_v < 90$ (mOsm/l.)

This solution may be extremely dilute and in the opposite direction to salt movement.

Active transport of solute: $135 < M_s/J_v < \infty$ (mOsm/l.)

Since a typical observed value of (M_s/J_v) is $(10^{-6}$ mole $cm^{-2}hr^{-1}/2$ mg $cm^{-2}hr^{-1})$ $= 10^3$ mOsm/l., Diamond's theory may indicate that water transport is passive and coupled to the sodium 'pump', but the validity of this is highly dependent on the value for σ_{NaCl}.

In applying Diamond's criteria to frog skin the importance of a precise determination of σ_{NaCl} must be stressed and, in fact, the meaning of this parameter for a composite membrane like frog skin is problematical since its measurement imposes experimental conditions likely to alter the normal passive properties of the membrane in series. Recently Grim (1963) has criticized Diamond's method (used in this study) of measuring σ_{NaCl} because it assumes that water transport is passive; however, Diamond (1962a) produced some evidence supporting this assumption. The determination of σ_{NaCl} for frog skin is too inaccurate to decide whether or not metabolic poisoning alters its magnitude.

From this theoretical standpoint, there is no substantial support for the view that the flows of water and sodium are linked, but Diamond's theory previously proved successful in describing an epithelial membrane with symmetrical permeability characteristics and a neutral NaCl 'pump' whereas frog skin is an asymmetrical structure with an active transport of sodium ions. A proper description of this phenomenon in frog skin may follow only when this theoretical treatment is applied to the individual epithelial membranes.

From the experimental standpoint, attempts to relate the two flows have been inconclusive. This is particularly the case where I and non-osmotic flow have been

observed simultaneously after inhibition and stimulation of the active sodium 'pump'.

In one of the experiments recorded in this chapter on the effects of Ouabain it appears at first sight that stoppage of sodium transport does not produce cessation of water transport. But the issue is complicated by the permeability changes taking place at the outer and inner membranes. MacRobbie and Ussing (1961) reported a general decrease in the permeability of the outer membrane to NaCl and of the inner membrane to KCl. The latter permeability change decays after one hour leaving the outer membrane still 'tight' to NaCl while the cells begin to shrink. I also observed a shrinkage of frog skin within two hours after the addition of Ouabain; if the water, leaving the skin during this phase, was taking the pathway of least resistance, i.e. the more permeable inner membrane, then this would be recorded as a net influx of 2.2 mg $cm^{-2}hr^{-1}$. This spurious flow and the other transient changes in passive properties of the skin make a simple explanation of this experiment impossible.

2.43 Thermo-osmosis

Spanner (1954) brought to notice the important point that a permanent temperature gradient across a membrane produces a net flow of water unless a back-pressure is allowed to build up to reduce this flow eventually to zero. Capraro and Garampi (1956) found that the Q_{10} for water absorption in frog skin was 2; with this value it is possible to calculate the temperature difference required to produce the water flow -- this 1.4×10^{-2} °C. Therefore, there must be a temperature gradient of about 0.7°C cm^{-1} across the skin and this will cause a heat flow of 10^{-3} cals $cm^{-2}sec^{-1}$ across this tissue if its thermal conductivity is the same as that of water (1.4×10^{-3}cal $sec^{-1}cm^{-1}degree^{-1}$). The skin must compensate, in

theory, for this heat flow by supplying energy from its metabolic processes, but the metabolic rate * of frog skin is not large enough (6×10^{-6} cal sec^{-1} cm^{-2}) to maintain this temperature gradient. Objection may be raised to the thermal conductivity value taken for frog skin; calculation shows that metabolism could balance the heat flow across the skin if the thermal conductivity were about 10^{-5} cal $sec^{-1} cm^{-1} degree^{-1}$. Only gases are such good thermal insulators and, therefore, thermo-osmosis cannot be accepted on energetic grounds.

2.44 Electro-osmosis

Electro-osmosis is the flow of water often observed when an electrical current is passed through a charged membrane. This mechanism has often been invoked to explain anamolous biological water flows e.g. in the intestine (Parsons and Wingate, 1958), plant roots (Spanner, 1958). Despite the frequency of this postulation electro-osmotic water flow has been observed only once in biological membranes -- in a plant cell, Nitella (Fensom and Dainty, 1963). These workers, assuming in their experiments that the current passing through the plant cell was entirely carried by positive ions, observed an electro-osmotic transport of water through Nitella of about 100 water molecules per ion. They concluded from the magnitude of the electrical currents driven by 'ion pumps' through the cell membranes that the electro-osmotic flow was not sufficient to explain the normal high

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* This is calculated from Zerahn's (1961) observations of the oxygen consumption of frog skin. He found that an area of 7 cm^2 consumed oxygen at a rate of 6 μequiv./hr. The calorific value of one equivalent of oxygen is taken as 25,000 cal.

turgor pressure of these cells.

In frog skin the apparent electro-osmotic efficiency is not large enough to account for the normal range of non-osmotic water transport; large electrical current densities (40 - 200 $\mu A\ cm^{-2}$) are necessary for the production of water flows in the range (1 - 5 $mg\ cm^{-2} hr^{-1}$) normally found. The simplest conclusion is that non-osmotic water transport may be partially electro-osmotic in character and that there exists another component of water flow moving independently of sodium transport. This latter component is still manifest, for example, when choline Ringer bathes the outside of the skin.

However, electro-osmosis can be established only when equality between the streaming potential 'cross-coefficient' and the electro-osmotic 'cross-coefficient' is demonstrated. Expressed mathematically this relation is:

$$\left(\Delta V / \Delta p\right)_{\Delta\pi, I} = \left(J_v / I\right)_{\Delta\pi, \Delta p}$$

where ΔV is the electrical potential difference.

Δp the pressure difference in a streaming potential experiment.

I the electric current,

J_v the volume flow in an electro-osmosis experiment, and

$\Delta\pi$ the osmotic pressure difference.

The subscripts indicate the flows or forces held at zero.

Streaming potentials have never been observed in frog skin and, furthermore, the composite membrane nature of frog skin complicates the definition of proper criteria for the demonstration of streaming potential and electro-osmotic flow. Thus it cannot be claimed that the existence of electro-osmosis in frog skin has been proved conclusively.

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CHAPTER THREE

THE NATURE OF WATER TRANSPORT ACROSS FROG SKIN

II. THEORETICAL MODEL

3.1 INTRODUCTION

Experiments reported in Chapter Two support the conclusions of several workers (Kirschner et al., 1960; Capraro and Marro, 1963) that there exists an net inward transport of water across frog skin conditionally unrelated to active transport of sodium. Curran and McIntosh (1962) and Ogilvie et al. (1963) studied net volume flow across a barrier (analogous to epithelial membranes) composed of two different membranes arranged in series and separated by a closed compartment. They observed a net volume flow across this system against a water activity gradient under certain conditions and suggested that this phenomenon could be explained in terms of the different properties of the membranes and the concentration and pressure gradients across the membranes. The aim in this chapter is to offer a similar theory describing the non-osmotic water flux across frog skin. The subsequent discussion relies much on the papers of Kedem and Katchalsky (1961, 1962) and Dainty (1963).

The three parameters determining the passive properties of a membrane with respect to transport of water and non-electrolyte solutes are σ, ω and L_p (see 1.21). Mathematical expressions for σ have been calculated for two special models by Kedem and Katchalsky (1961) and by Dainty and Ginzburg (1963) (see 1.22) and both formulae are formally identical to:

$$\sigma = 1 - \omega_s \bar{V}_s / L_p \quad - \alpha_s \qquad (1)$$

where α_s expresses a direct interaction between solute and water passing through water-filled pores in the membrane.

In the case where no water-filled pores exist in the membrane equation (1) becomes:

$$\sigma = 1 - \omega_s \bar{V}_s / L_p \qquad (2)$$

If the solute does not penetrate the membrane equations (1) and (2) reduce to $\sigma = 1$.

So far the discussion has dealt with the permeation of non-electrolytes in membranes but Kedem and Katchalsky (1961) have shown in the case of salt permeation that, although σ is dependent on concentration, the equations for σ are formally identical to (1) with the proviso that the membrane does not have a high charge density. Making this latter assumption I have produced a simple theory of water transport across frog skin.

3.2 THEORY

There are certain features of the model of frog skin, suggested by Koefoed-Johnsen and Ussing (1958), which indicate that there may exist driving forces for the net influx of water. Their model implied a selective permeability of the outer surface of the skin to sodium ions while the inner was selective for potassium ions. Both membranes were permeable to chloride ions. Therefore, it is conceivable that the reflection coefficients of the outer and inner membranes for NaCl and KCl respectively may be significantly less than unity.

The following basic assumptions are made:

(1) The 'ion-pumps' maintain a high constant concentration of potassium ions and a relatively negligible sodium concentration between the outer and inner boundaries of the skin.

(2) The ability of the outer membrane to discriminate between KCl and water makes the reflection coefficient for KCl unity.

(3) Similarly the reflection coefficient of the inner membrane for NaCl is unity.

In the Appendix a model is considered in which assumptions (2) and (3) do not necessarily hold.

Consider the model system in Fig. 1. The outer and inner membranes of the

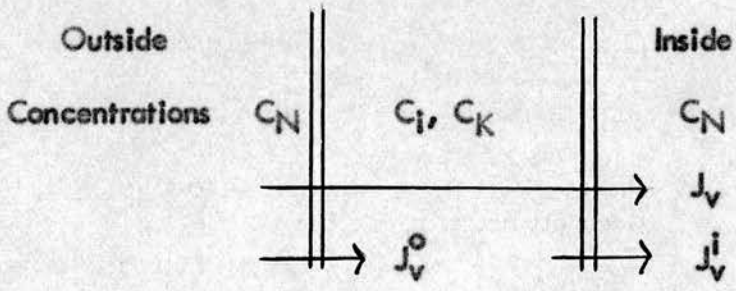


Fig. 1. Basic system of membranes. Symbols explained in the text.

skin enclose a fluid having an osmolarity, C_i, of impermeable potassium salt and an osmolarity, C_K, of KCl. Both surfaces of the skin are bathed by identical NaCl Ringer solutions, osmolarity C_N. The concentrations (C_i, C_K, C_N) should be expressed preferably as osmolalities. The volume flow across the two membranes, when no hydrostatic pressure differences exist, are:

$$J_v^o = L_p^o RT\left[C_K + C_i - \sigma_N^o C_N\right] \tag{3}$$

$$J_v^i = L_p^i RT\left[C_N - C_i - \sigma_K^i C_K\right] \tag{4}$$

where: J_v^o = Net volume flow across outer membrane ($cm^3 cm^{-2} sec^{-1}$)

J_v^i = Net volume flow across inner membrane

L_p^o = Hydraulic conductivity of outer membrane ($cm\ sec^{-1} atm^{-1}$)

L_p^i = Hydraulic conductivity of inner membrane

C_K = Osmolarity of KCl in central compartment ($moles\ cm^{-3}$)

C_i = Osmolarity of impermeable potassium salt in central compartment

C_N = Osmolarity of NaCl in external Ringer solutions

σ_N^o = Reflection coefficient of outer membrane for NaCl

σ_K^i = Reflection coefficient of inner membrane for KCl

Equating J_v^o and J_v^i to the net water flux, J_v, across the system, and letting $\lambda = (C_i/C_K)$, then equations (3), (4) become:

$$L_p^o\left[C_K(1+\lambda) - \sigma_N^o C_N\right] = L_p^i\left[C_N - C_K(\sigma_K^i + \lambda)\right] = J_v/RT \tag{5}$$

From equation (5), an equation for C_K follows

$$C_K = \frac{C_N\left(L_p^o\sigma_N^o + L_p^i\right)}{L_p^o + L_p^i\sigma_K^i + \lambda\left(L_p^o + L_p^i\right)} \tag{6}$$

Substituting for C_K into equation (5), then

$$J_v = \frac{L_p^o L_p^i RTC_N\left[1 - \sigma_N^o\sigma_K^i + \lambda(1-\sigma_N^o)\right]}{L_p^o(1+\lambda) + L_p^i(\sigma_K^i + \lambda)} \tag{7}$$

MacRobbie and Ussing (1961) found that (L_p^i/L_p^o) was about 20 and for this model I have taken $L_p^o = 4 \times 10^{-7}$ and $L_p^i = 8 \times 10^{-6}$ cm sec^{-1}atm^{-1}. From experiments on the diffusion of KCl out of the epithelial cells Ussing (1960) concluded that at least 30% of the osmolarity of the cells was due to KCl. This implies $0 < \lambda < 3$; unfortunately there have been no direct measurements of cellular concentrations of potassium and chloride ions, although MacRobbie and Ussing estimated the cellular concentration of chloride ions was about 50 mM/l. Assuming these values for L_p^o and L_p^i then J_v is plotted against σ_K^i for four values of σ_N^o (Fig. 2) at $\lambda = 3$ and $\lambda = 1$. Both external solutions are taken as Ringer ($C_N = 1.8 \times 10^{-4}$ mole cm^{-3}).

Besides J_v it is also interesting to know what values of C_K are implied by the model. Re-arranging equation (6) and substituting $(L_p^i/L_p^o) = 20$, then

$$C_K = \frac{C_N(\sigma_N^o + 20)}{1 + 20\sigma_K^i + 21\lambda} \tag{8}$$

Choosing values of $\lambda = 3$ and $\lambda = 1$, C_K is plotted (for extreme values of σ_N^o) against σ_K^i (Fig. 3). In the extreme case $\lambda = 0$, (i.e. $C_i = 0$), C_K lies in the range 3780 - 186 mOsm/l.

Fig. 2. The net water influx, J_v, across the membrane-system as a function of σ_K^i for $\lambda = 1$ and $\lambda = 3$. The values of σ_N^o (0.1, 0.4, 0.8 and 1) used are shown on the corresponding curves. The ordinate axis on the right of the diagram expresses the predicted J_v in conventional units.

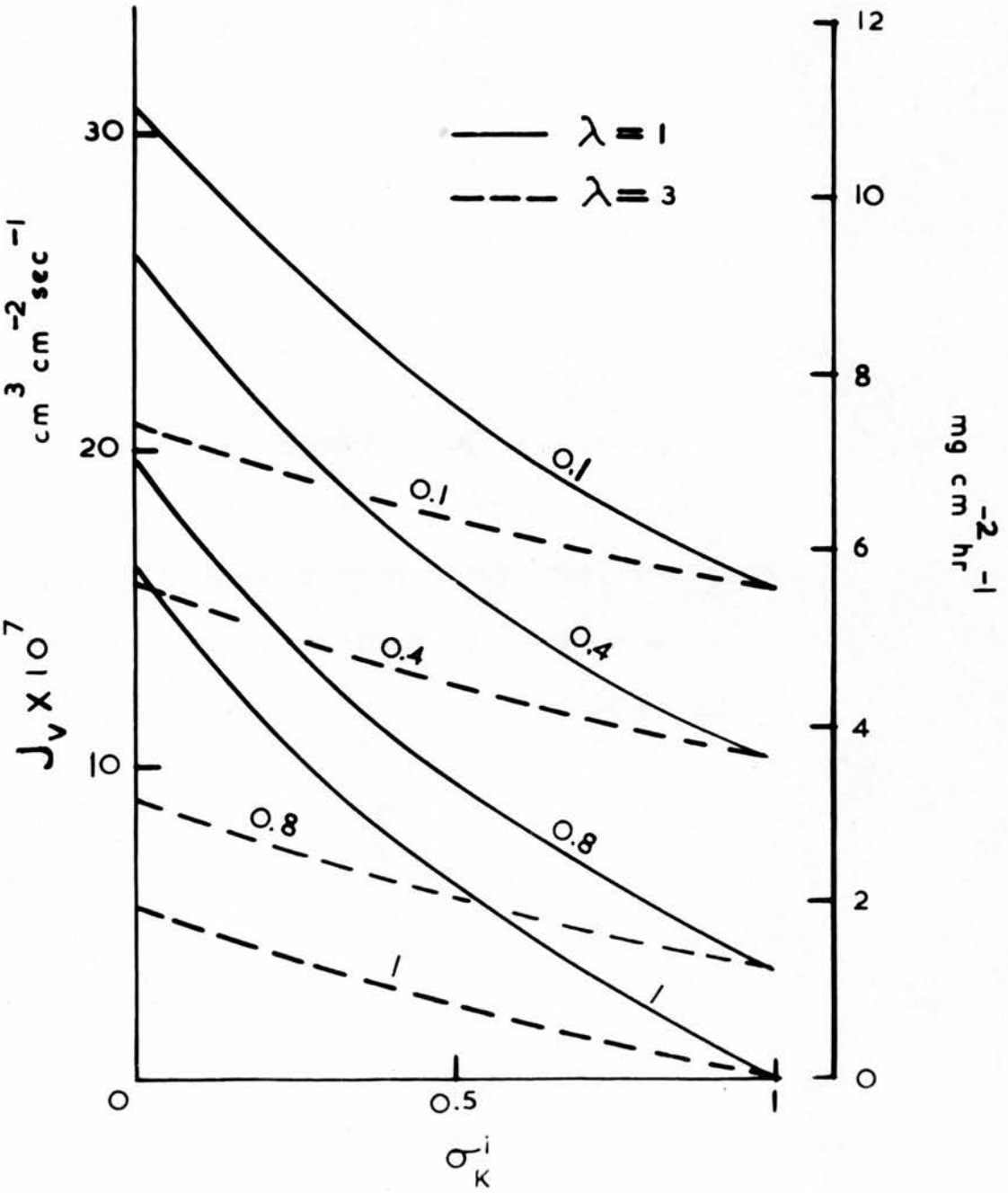
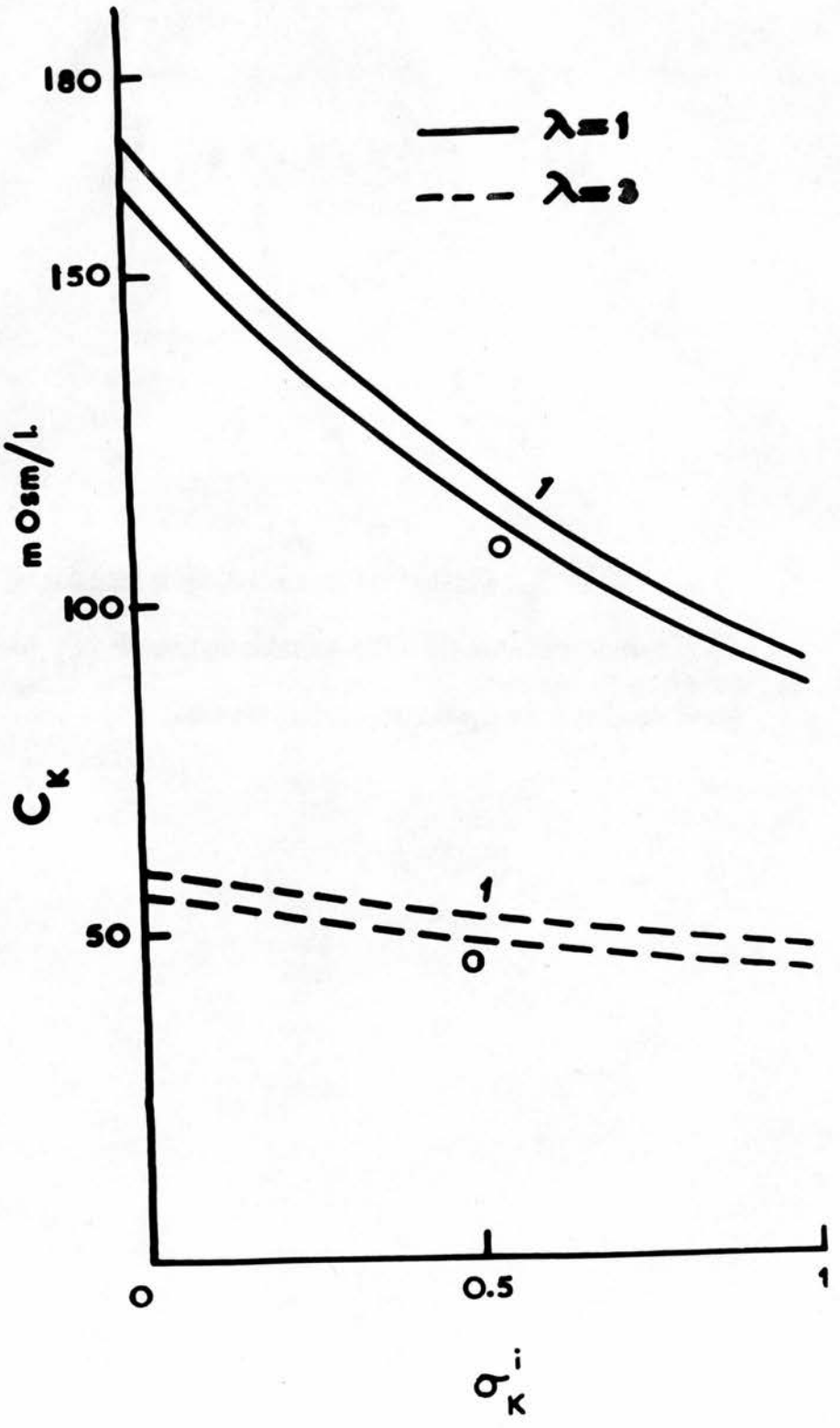

λ = 1
λ = 3
0.1
0.1
0.4
0.4
0.8
0.8
1
1
30
20
10
0
0.5
1
12
10
8
6
4
2
0
$J_v \times 10^7$
$cm^3\ cm^{-2}\ sec^{-1}$
$mg\ cm^{-2}\ hr^{-1}$
σ_K^i

Fig. 3. The values of C_K, implied by the model, as a function of σ_K^I for $\lambda = 1$ and $\lambda = 3$. The extreme values of σ_N^o (0 and 1) used are shown on the corresponding curves.


180
150
100
50
mOsm/L
C_K
λ=1
λ=3
1
0
1
0
0
0.5
1
σ_K^i

3.3 DISCUSSION

The theoretical model described above makes three correct predictions about the nature of water transport across frog skin. First, the model quantitatively predicts a net volume flow, J_v, of the experimentally observed magnitude and direction across this system. Secondly, J_v is not directly coupled to active sodium transport as it would be if it were completely electro-osmotic; however, J_v does depend on metabolically-driven 'ion-pumps' maintaining a constant intra-cellular medium. Finally, the model predicts the existence of an inward flow of water when inert Ringer (sodium replaced by impermeable cation) bathes the external surface; in this case $\sigma^o_N = 1$ and J_v is reduced. Moreover, the nature of intra-cellular medium implied by this hypothesis agrees with some experimental facts. Whittembury (1962) found an osmolarity of 205 mOsm/l. for the osmotically impermeant contents of the cells (i.e. C_i = 205 mOsm/l.) and, as MacRobbie and Ussing's estimate of the chloride ion concentration was 50 mM/l., it appears that C_K = 100 mOsm/l. and $\lambda = 2$ approximately. The model permits these conditions at values of σ^o_N and σ^i_K less than unity (Fig. 3).

Despite such success there is reason to doubt the validity of this hypothesis: the crucial objection to this theory falls on the assumption that both σ^o_N and σ^i_K are less than unity. Prima facie there appears to be some justification for this supposition on the basis of the high selectivity of the outer and inner membranes to NaCl and KCl demanded by Koefoed-Johnsen and Ussing's (1958) description of the frog skin's electrical potential. Recently, however, Janáček (1963) has discovered evidence repudiating such high selectivity of this membrane system. Despite these new facts it remains conceivable that $0 < \sigma^o_N < 1$ and

$0 < \sigma_K^i < 1$; this possibility arises from the nature of the formula for the reflection coefficient.

Kedem and Katchalsky (1961) have shown that σ for the permeation of uni-univalent salt through a charged membrane is formally identical to equation (1). With this in view, the assumption that $P^o_{NaCl} \gg P^o_{KCl}$ for the outer membrane can be replaced by a more specific one i.e. $\left[\alpha^o_{NaCl} + (P^o_{NaCl} \bar{V}_{NaCl} / RT\, L^o_p) \right] > 0$ and $\left[\alpha^o_{KCl} + (P^o_{KCl} \bar{V}_{KCl} / RT\, L^o_p) \right] = 0$. Corresponding assumptions can be made for the inner membrane. Unfortunately there is no quantitative evidence to support this view of the membranes except for a calculation of the magnitude of $(P_{NaCl} \bar{V}_{NaCl} / RT\, L_p)$. Morel (1958) taking into account the electrical driving forces across the membranes found the sodium permeabilities of these outer and inner boundaries to be about 10^{-4} and 3×10^{-7} cm sec^{-1} respectively. Curran et al. (1963) studying the kinetics of transport of isotopic sodium across the skin have estimated the outer membrane's permeability to sodium as 10^{-5} cm sec^{-1}. Taking for the outer membrane, $L^o_p = 4 \times 10^{-7}$ cm $sec^{-1} atm^{-1}$ and $P^o_{NaCl} = 10^{-5}$ cm sec^{-1}, then $(P^o_{NaCl} \bar{V}_{NaCl} / RT\, L^o_p) = 0.02$. For the inner membrane, $L^i_p = 8 \times 10^{-6}$ cm $sec^{-1} atm^{-1}$ and $P^i_{NaCl} = 10^{-7}$ cm sec^{-1}, give $(P_{NaCl} \bar{V}_{NaCl} / RT\, L^i_p) = 10^{-5}$. Absence of data on potassium permeabilities makes similar calculations impossible.

Until precise determinations of σ^o_N and σ^i_K are performed, however, this model can derive support only from its predictions about the behaviour of non-osmotic flow in certain experiments. For instance, an increase in L^o_p should produce an increase in J_v and this seems to have been observed experimentally with antidiuretic hormone (see 2.33).

Despite the serious objection that both σ^o_N and σ^i_K may equal unity, the

model may aid the interpretation of experiments on the net influx of water across frog skin; moreover, this hypothesis provides a simple quantitative description of the water absorption process -- the non-osmotic flow of water across frog skin is paradoxically an osmotic flow.

3.4 APPENDIX

The basic assumptions about the reflection coefficients in the theoretical model (see 3.2) can be expressed mathematically as follows:

$$0 < \sigma_N^o < 1 \;;\; 0 < \sigma_K^i < 1 \;;\; \sigma_K^o = \sigma_N^i = 1 \qquad (1)$$

where: σ_N^i = reflection coefficient of inner membrane for NaCl

σ_K^o = reflection coefficient of outer membrane for KCl

The assumption, $\sigma_K^o = \sigma_N^i = 1$, is now replaced by:

$$0 < \sigma_K^o \leq 1 \;\;;\;\; 0 < \sigma_N^i \leq 1 \qquad (2)$$

Moreover, it is assumed that both σ_K^o and σ_N^i are larger than either σ_N^o or σ_K^i.

The volume flows across the outer and inner membranes, when no hydrostatic pressure differences exist, are:

$$J_v^o = L_p^o RT\left[\sigma_K^o C_K + C_i - \sigma_N^o C_N\right] \qquad (3)$$

$$J_v^i = L_p^i RT\left[\sigma_N^i C_N - C_i - \sigma_K^i C_K\right] \qquad (4)$$

Again equating J_v^o and J_v^i to the net water flux, J_v, across the system, then equations (3), (4) become:

$$L_p^o\left[C_K(\sigma_K^o+\lambda) - \sigma_N^o C_N\right] = L_p^i\left[\sigma_N^i C_N - C_K(\lambda+\sigma_K^i)\right] = J_v/RT \qquad (5)$$

From equation (5), an equation for C_K follows

$$C_K = \frac{C_N\left(L_p^o \sigma_N^o + L_p^i \sigma_N^i\right)}{L_p^o\left(\sigma_K^o + \lambda\right) + L_p^i\left(\sigma_K^i + \lambda\right)} \tag{6}$$

Substituting for C_K back into equation (5) gives:

$$J_v = \frac{L_p^o L_p^i RTC_N\left[\sigma_N^i\left(\sigma_K^o + \lambda\right) - \sigma_N^o\left(\sigma_K^i + \lambda\right)\right]}{L_p^o\left(\sigma_K^o + \lambda\right) + L_p^i\left(\sigma_K^i + \lambda\right)} \tag{7}$$

Equation (7) predicts a net influx of water provided that

$$\sigma_N^i\left(\sigma_K^o + \lambda\right) > \sigma_N^o\left(\sigma_K^i + \lambda\right) \tag{8}$$

The relation (8) is completely compatible with the assumptions that have been made about σ_N^i and σ_K^o.

Provided that relation (8) holds then the magnitude of J_v can be discussed in the following particular case. Suppose that $C_N = 2C_K = 2 \times 10^{-4}$ mole cm^{-3}, $\lambda = 1$, $L_p^o = 4 \times 10^{-7}$ and $L_p^i = 8 \times 10^{-6}$ cm sec^{-1}atm^{-1}. Then σ_K^i is eliminated from equations (6) and (7) to give:

$$J_v = 10^{-6}\left(1 + \sigma_K^o - 2\sigma_N^o\right) \qquad \text{cm}^3\,\text{cm}^{-2}\,\text{sec}^{-1} \tag{9}$$

Implicit in the assumptions about σ_K^o is the particular one:

$$0 < \sigma_N^o < \sigma_K^o \leqslant 1$$

Hence J_v is given by:

$$10^{-6}\left(1 - \sigma_N^o\right) < J_v \leqslant 2 \times 10^{-6}\left(1 - \sigma_N^o\right) \tag{10}$$

The relation (10) permits values of J_v within the experimentally observed range.

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CHAPTER FOUR

OSMOTIC REGULATION IN TELEOSTS

4.1 INTRODUCTION

Studies on the osmotic relations of aquatic animals began about 1900 with investigations on the composition of their body fluids measured mainly by freezing-point depression. The results showed that whereas most marine invertebrates were in osmotic equilibrium with their environment, this was not generally true of the marine vertebrates and of fresh-water animals. Osmotic regulation in animals involves the maintenance of the body fluid in a steady state different from the environmental composition; invariably body fluid and environment are separated by a bounding membrane divided into special regions such as integument, gut, excretory systems and gills. These particular organs are intimately connected with the osmo-regulatory processes and are the foci of studies on the mechanisms of regulation.

The dominant aquatic animals of to-day are the teleosts; they have become adapted to almost every aquatic medium and this adaptive ability depends to a large measure on their osmo-regulatory powers. The study of osmotic regulation in teleosts has been reviewed by Black (1951, 1957).

In fresh-water fish the blood has a concentration of 130 - 170 milliosmolar (predominantly sodium chloride) and there is a large flow of dilute urine presumably to balance the osmotic influx of water across the gill and oral membranes. The kidneys of these fish have well-developed nephrons and the urine produced after re-absorption of salts in the tubules is not as dilute as the external medium -- hence there is a salt loss. Salts are replaced either by the ingestion of food or by absorption processes in the gills. Krogh (1937) demonstrated the absorption of ions in seven species of fresh-water teleost; he found evidence for the absorption of chloride, bromide, sodium and, in one case, calcium ions. Meyer (1951, 1952) found that the gills of the goldfish absorbed sodium ions and that mercuric ion (10^{-5} Molar) completely

inhibited the uptake of sodium in the gills.

In the ocean the osmotic problem of teleosts is to conserve water and exclude salts because their blood is considerably hypotonic to sea water. To this end, urine flow is small and always hypo-osmotic to the blood. Smith found that marine fish, unlike fresh-water ones, swallow large quantities of the medium. As the sea water passes down the intestine, water and some ions are absorbed. The osmotic concentration decreases as the medium proceeds towards the colon and the absorbate is hypertonic to blood as shown for Lophius (Smith, 1932). In the gut, some of the luminal electrolytes, particularly magnesium and sulphate, are lost in the faeces; the urine solutes are also largely magnesium, calcium, sulphate and phosphate, while most of the sodium, potassium and chloride must be excreted extra-renally. In view of the reduced urine output it is not surprising to find less well developed kidneys in marine teleosts than in fresh-water ones (Marshall, 1934) and in Lophius, for example, glomeruli are absent in the adults. That the gills are the root of extra-renal excretion of sodium chloride was clearly shown by perfusion of a heart-gill preparation of the eel (Keys, 1933). Keys found that chloride was secreted into sea water and that, when the chloride concentration of the perfusion fluid was increased, the excretion rate increased.

Bodies of water, such as estuaries and saline pools, where the salt content lies between that of sea water and fresh water, support a small varied fauna. Perhaps the most interesting brackish water animals are some teleosts, especially migratory forms like the eel and salmon, which must not only possess the osmo-regulatory mechanisms of marine and fresh-water fish but also must exert a considerable control over them.

The bulk of the support for these hypotheses of osmotic regulation in teleosts rests on *in vivo* measurements of fluxes of water and ions. Although this circumstantial evidence seems convincing, proof of its validity will follow only after thorough *in vitro* investigations of the epithelia involved in the regulation processes. In this respect, previous studies on the frog skin, on the isolated intestine and, recently, on the isolated crustacean gill (Croghan *et al.*, to be published) point the way to future progress in this field.

This Chapter records the results of *in vivo* measurements of the electric potential differences existing between the blood of teleosts and the external media. These studies were undertaken to determine the nature of ionic movements across the gills in these conditions. Investigations on the nature of sodium and water fluxes across the isolated intestine of a marine teleost are reported also.

4.2 THEORETICAL BASIS

The equations governing the exchange of radioactive ions between two compartments have been outlined by Ussing (1949) and others.

If a compartment (e.g. a tissue sac filled with fluid) containing no radioactive ions is placed in contact with a large volume of solution in which the total external concentration (μ.equiv. cm^{-3}) of the ion species, under study, is C_o and the external concentration of labelled ions is C_o^* (counting rate/cm^3), then the initial rate of entry of labelled ions is:

$$\frac{dC_i^*}{dt} = J_{in} \cdot \frac{A}{v} \cdot \frac{C_o^*}{C_o} \qquad (1)$$

where A is the surface area (cm^2) across which ion exchange takes place, v is the compartment volume (cm^3), C_i^* is the internal concentration of labelled ions and J_{in} is the influx (μ.equiv. $cm^{-2}\ hr^{-1}$) of the ion. After some time when radioactive ions are crossing the surface in both directions, the rate of change of internal radioactivity is:

$$\frac{dC_i^*}{dt} = J_{in} \cdot \frac{A}{v} \cdot \frac{C_o^*}{C_o} - J_{out} \cdot \frac{A}{v} \cdot \frac{C_i^*}{C_i} \qquad (2)$$

where C_i is the internal concentration of the ion species and J_{out} is the efflux of the ion from the compartment. In a washing-out experiment, in which the radioactive ion is not allowed to accumulate in the external medium, C_o^* may be neglected and the solution of equation (2) is:

$$C_i^* = (C_i^*)_{t=0} \cdot \exp\left(-J_{out} \cdot \frac{A}{v} \cdot \frac{t}{C_i}\right) \qquad (3)$$

where $(C_i^*)_{t=o}$ is the initial concentration of labelled ion in the compartment.

If $\ln C_i^*$ is plotted against time (t) the graph will be a straight line of slope ($- J_{out} A/vC_i$) and this allows calculation of the efflux provided C_i, v and A are known. The influx may be calculated from the uptake (over a period short enough for C_i^* to be considered negligible) by equation (1).

Generally, fluxes of ions across biological membranes are driven by at least two physical forces produced by the chemical and electrical potential gradients. There are other possible driving forces; for example, if water is flowing through the membrane, a viscous drag may be exerted on the ions (Ussing, 1952). Neglecting this force it can be shown that, if there is no electrochemical potential difference for an ion j between two solutions separated by a membrane, then

$$\Delta V_j = \frac{RT}{z_j F} \cdot \ln\left(a_j^o / a_j^i\right) \qquad (4)$$

where ΔV_j is the electrical potential difference (outside taken as reference i.e. $\Delta V_j = V_j^i - V_j^o$) across the membrane in volts, a_j^o and a_j^i are the chemical activities of the ion j in the outer and inner solutions, z_j the algebraic valency, F, the Faraday, R the gas constant and T the absolute temperature. Equation (4) is the Nernst equation for the transmembrane potential difference when the ion j is in passive equilibrium. This is a dynamic equilibrium (often referred to as flux equilibrium) for the fluxes of ion j in the two directions will be equal. Generally, in biological systems the chemical activities are replaced by concentrations and the Nernst equation becomes:

$$\Delta V_j = \frac{58}{z_j} \log\left(C_j^o / C_j^i\right) \qquad \text{millivolts} \qquad (5)$$

at ordinary temperatures.

Teorell (1949) and Ussing (1950) have shown that the passive movement of ions across membranes is governed by the relation

$$\frac{J_{in}}{J_{out}} = \frac{C_j^{\circ}}{C_j^{i} \exp(z_j F \Delta V / RT)} \qquad (6)$$

where J_{in} and J_{out} are the passive influxes and effluxes of the ion $\underline{j}$ across a membrane and ΔV is the electrical potential difference in volts across the membrane. This expression follows from the fact that the ratio of passive fluxes is equal to the electrochemical activity ratio. When $J_{in} = J_{out}$, i.e. when there is flux equilibrium, equation (6) reduces to the Nernst equation (5).

Equation (6) forms an important criterion for independent passive ion movement. Deviations from this equation do not, however, invariably indicate active transport of an ion. In the case where the ratio of influx to efflux of an ion is closer to unity than would be expected from the electrochemical potential difference then active transport of the ion is likely unless this deviation persists after metabolism has stopped. Ussing (1952) proposed an 'exchange diffusion' mechanism to explain such data. In this model the ions cross the membrane in combination with a 'carrier' which is always saturated with ions so that a perfect one-to-one exchange occurs at the membrane faces and the exchange flux is limited only by the number of 'carriers' and their rate of movement across the membrane. Moreover, an 'anomolous' flux ratio may result from the interactions of the flowing solute particles among themselves (Hodgkin and Keynes, 1955; Meares and Ussing, 1959).

4.3 METHODS

Animal species from the three principal aquatic habitats -- marine, brackish and fresh water -- were used. Marine teleosts, Cottus scorpius, were obtained from Pittenweem, Fife, while the euryhaline teleosts, Blennius pholis, were collected from Port Seton, the Firth of Forth. The fresh-water goldfish, Carassius auratus, were bought from a commercial supplier. In vivo potential measurements were performed on the blennies and goldfish in the range 14 - 19 °C. The net fluxes of water and sodium were measured in the isolated small intestine of Cottus at room temperature in the Gatty Marine Laboratory, University of St. Andrews.

4.31 Ionic Concentration in Blood

The electrolyte levels in the blood of the blennies and goldfish were always measured on the conclusion of the potential measurements in the different media. After removing the animal from the medium it was killed by a sharp blow on the head, washed quickly with de-ionized water and pinned to a wooden block. Collecting pipettes had been made by drawing out 2 mm diameter glass tubing to a sharp tip and mounting each pipette on a piece of rubber tubing the other end of which was held in the mouth. The body wall was cut along the ventral surface from posterior to anterior and the heart punctured by the fine tip of the pipette. Blood rose into the tip by capillary attraction and by a small suction applied by mouth. Finally, some liquid paraffin was drawn into the pipette, the tip was sealed with wax and the sample was centrifuged. Samples of blood serum were drawn off from below the paraffin layer into micro-pipettes and suitably diluted for measurement against standard sodium chloride and potassium chloride solutions using an EEL flame-photometer. In this way duplicated sodium and potassium

concentrations in blood serum of the animals were determined. The accuracy of the sodium measurements was limited by the reading error on the instrument (maximum error was about 5%) and possible dilution of samples during withdrawal from the animals. The accuracy of the potassium measurements was exceedingly low due to almost inevitable haemolysis of red cells during centrifugation, and for this reason, these results are not quoted in detail.

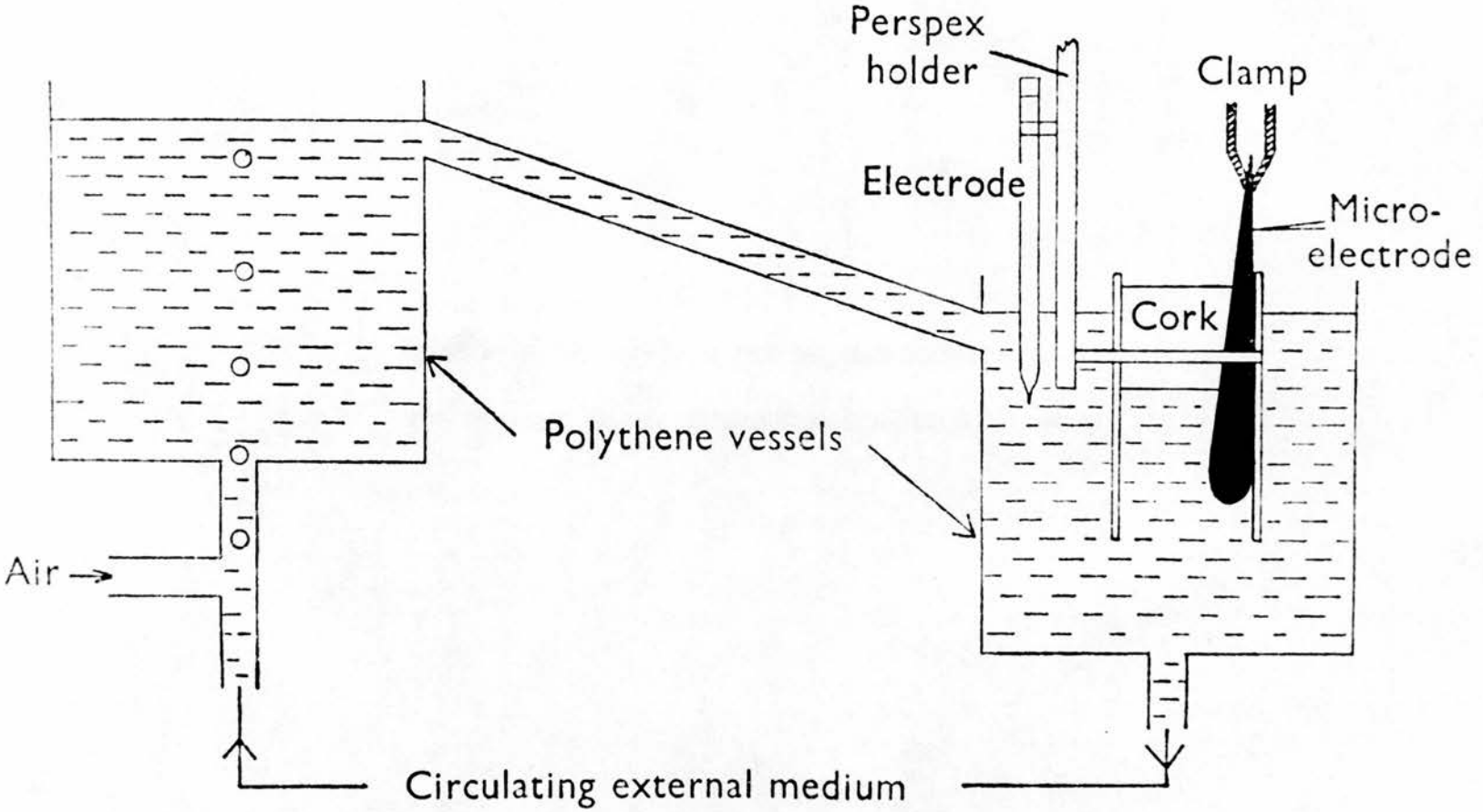
The chloride concentrations in blood serum were measured by the direct titration of serum volumes (diluted with 5% nitric acid) with 0.01 Molar silver nitrate solution and the end-point was determined potentiometrically. This method was originally used by Sanderson (1952) and modified successfully for smaller volumes by Ramsay, Brown and Croghan (1955) and Croghan (1958).

4.32 Potential Measurements

In the experiments on goldfish the external medium was an artificial pond-water containing 2 mM NaCl, 0.1 mM KCl and O.1 mM/l. $CaCl_2$. For the blennies, the external media were prepared by suitable dilutions of an artificial sea water (S.W.) (chief constituents: 470 mM sodium , 549 mM chloride and 10 mM/l. potassium) described by Hale (1958). The 150% S.W. was prepared by adding 235 mM sodium chloride to a litre of 100% S.W. Adult animals were used and their wet weights lay within the range 2.5 - 7 g.

The animals were individually transferred from a large well-aerated tank of sea water or pond water to the new external media in glass jars (also continuously aerated). The animals wereleft for at least 48 hours to acclimatize before the potential measurements were made. During the measurements the animal was clamped by the tail fin with its head and gills immersed in the medium (see Fig. 1), which was continuously aerated and circulated between two polythene vessels.

Fig. 1. The apparatus for the measurement of electric potential differences between blood and external medium.


Perspex
holder
Clamp
Electrode
Micro-
electrode
Cork
Polythene vessels
Air
Circulating external medium

The problem of holding the animal was solved by placing the animal in a vertically clamped 3 cm diameter thin perspex cylinder and filling the space between the animal and cylindrical surface above the head with cork. This ensured that there was little movement of the animal and also that no damage occurred to the gills. The perspex cylinder was drilled with many holes to allow adequate circulation of the aerated medium around the head region.

Micro-electrode tips were made by drawing out small pieces of 2 mm diameter glass tubing to a sharp point and were filled with 3 Molar KCl. One of these fine tips was attached by a rubber collar to the tip of a Pye $Hg/Hg_2Cl_2/Sat.$ KCl electrode and the potential difference between this and another $Hg/Hg_2Cl_2/Sat.$ KCl electrode, clamped in the external medium, was measured with a Radiometer pH Meter 3, Copenhagen. The potential difference was measured with both electrodes in the external solution to determine any asymmetry. Then the tail of the animal was sprayed several times with de-ionized water and blotted dry to reduce short-circuiting. The fine electrode was mounted on a Prior micro-manipulator and the tip was inserted under the skin of the tail region where it was assumed to be in contact with the body fluids; the potential difference ($\Delta V = V^b - V^m$, the superscripts i and o have been replaced by b, blood, and m, medium) was then measured at various time intervals. There was always some uncertainty as to whether or not the tip was in the blood, but on numerous occasions blood was observed at the point of insertion. On completion of the experiments the asymmetry between the electrodes was again measured in the medium and a correction applied to the observed steady value of the potential difference between blood and medium. In the measurements on the goldfish large variable tip potentials were found on removing the micro-electrodes from the animals. This

agrees with observations in similar electrical studies on plant and animal material where large tip potentials develop due to blockage of micro-electrodes with cell matter. To remedy this I injected (via an Agla micrometer syringe) about 0.2 μ l. of 3 Molar KCl into the rubber collar of the micro-electrode immediately after insertion. This apparently halted the blockage of tips and upon removal no large variable tip potentials existed.

In this way ($V^b - V^m$) was measured for the blenny in 10, 40, 100 and 150% S.W. and for the goldfish in artificial pond water. In all of the in vivo experiments ΔV could be measured to at least $\pm$ 0.5 millivolt (mV.). The sign convention used in this chapter is that the potential of the extra-cellular fluid of the animal has always been measured with the external medium as reference. The Nernst potentials have also been stated in the same convention.

In the in vitro experiments on the small intestine of Cottus ΔV was measured by silver-silver chloride electrodes connected to a Radiometer pH Meter 22, Copenhagen. The tissue was mounted in the gravimetric chamber apparatus fully described in 2.21 and 2.22.

4.33 Flux Measurements

In all of the flux experiments on the isolated intestine of Cottus the tissue was bathed on both sides by Ringer solution. Since there was no available information on the ionic concentrations in the blood of Cottus, a Lophius Ringer (modified from Young, 1933) was used. This Ringer contained 205 mM NaCl, 8 mM KCl, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, 2.3 mM $NaHCO_3$, 0.5 mM KH_2PO_4 and 2.8 mM/l. glucose. The pH was 7.2. This Ringer was considered to be satisfactory because the tissue respired continuously in it for periods of at least six hours. Respiration was followed in a Warburg apparatus.

The serosal to mucosal sodium fluxes were found by immersing normal filled sacs in a continuously aerated ^{22}Na-loaded Ringer and monitoring the sacs in a well-type scintillation counter at known times after immersion. Before the counting procedure the sacs were washed by four rapid successive immersions in three separate volumes of Ringer. This washing technique lasted less than 20 seconds and its efficiency was found by immersing two normal filled sacs in the radioactive Ringer for 60 seconds and then monitoring the sacs after the same washing. The specific activity of the sacs was about 1% of the specific activity of the radioactive Ringer. Values of the sodium flux were calculated from the equations for a two-compartment system. The sacs were then transferred to a large volume of aerated Ringer and the mucosal to serosal fluxes of sodium were determined from the decrease in specific activity of the sacs with time.

Net water transport was determined by the change in weight of normal and everted sacs, filled with Ringer, during one hour incubation periods in Ringer, and also by the gravimetric chamber technique described in 2.21.

In all of the flux experiments the tissue was always equilibrated with Ringer for one hour before commencing the measurements.

4.4 RESULTS

4.41 Experiments on Goldfish

The determinations of sodium and chloride concentrations in the blood serum of 12 goldfish gave a mean ± SE for sodium of 82 ± 2 mM/l. (23 measurements) and for chloride of 74 ± 2 mM/l. (22). Table 1 gives the Nernst potentials for these ions and the mean ± SE of $(V^b - V^m)$ for goldfish in artificial pond water.

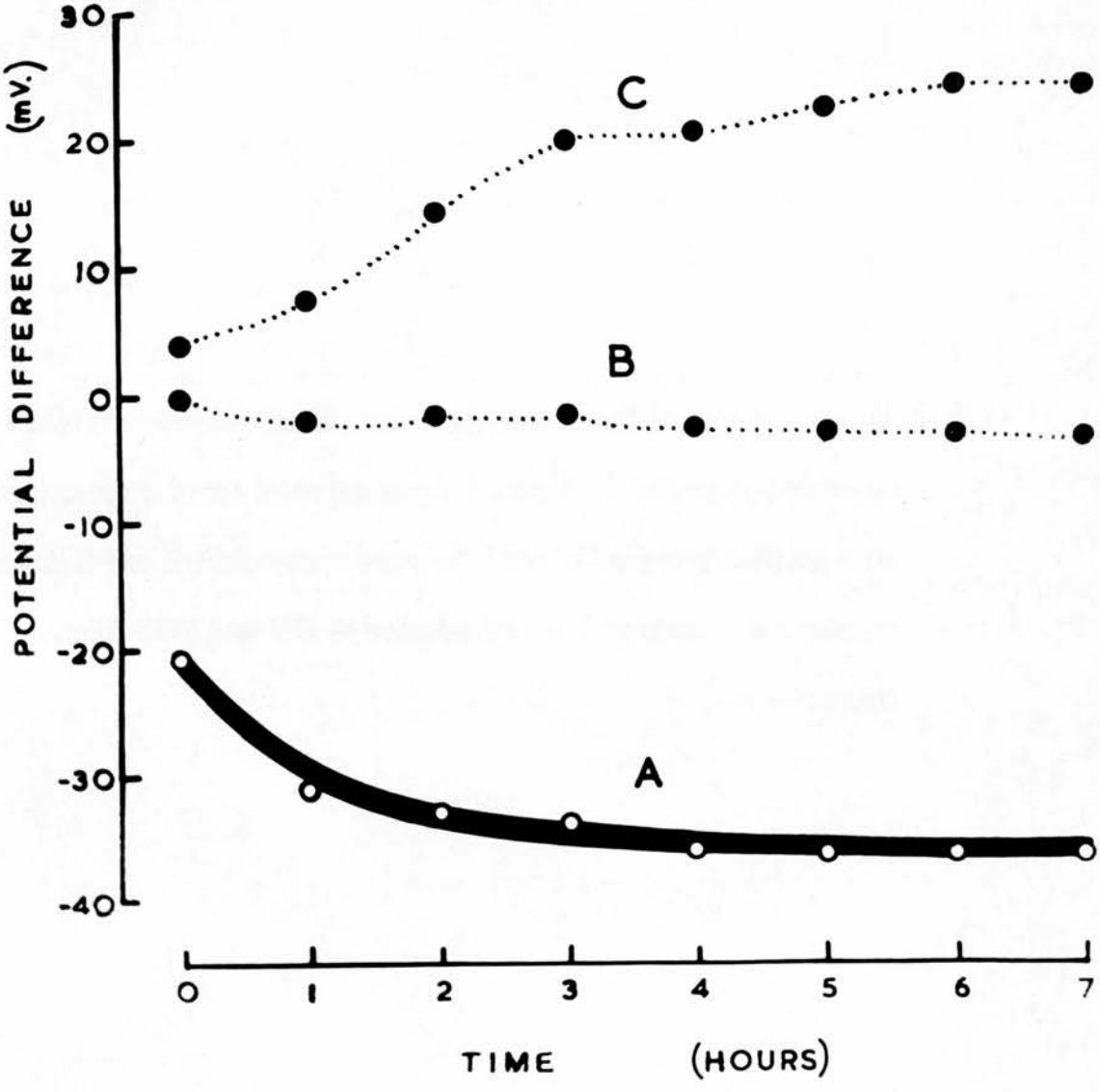
TABLE 1

SODIUM AND CHLORIDE NERNST POTENTIALS AND $(V^b - V^m)$

ΔV_{Na} Mean ± SE	ΔV_{Cl} Mean ± SE	$(V^b - V^m)$ Mean ± SE
- 94 ± 1 (mV.)	+ 87 ± 1 (mV.)	- 38 ± 3 (mV.)

In all experiments $(V^b - V^m)$ did not reach a steady value till several hours after the insertion of the micro-electrode. Invariably the potential difference increased in magnitude almost linearly in the first few hours and then tended asymptotically to a final steady value (see Fig. 2). This general type of variation in $(V^b - V^m)$ might be caused by some electrical short-circuiting across the body surface between the electrodes. This short-circuiting would be expected to decrease as a function of time as the body surface between the point of insertion of the micro-electrode and the liquid surface progressively dried, and also it would be expected to be more effective in the more concentrated media because of the higher electrical conductivity. This effect was actually observed in the potential measurements on the blenny. On completion of the potential measurements the animals appeared normally active and therefore it was

Fig. 2. The time course of the electric potential difference between blood and external medium. Curve A shows a typical set of observations on a goldfish immersed in artificial pond water while B and C show measurements made on blennies adapted to 10% and 100% S.W. respectively.


POTENTIAL DIFFERENCE (mV.)
30
20
10
0
-10
-20
-30
-40
C
B
A
0
1
2
3
4
5
6
7
TIME (HOURS)

assumed that the ($V^b - V^m$) had been measured while the gills were functioning normally. Generally, the measurements were made at 20 minute intervals and the potential difference was judged to be a steady value if it did not change by more than 1 mV. in one hour. During the course of some experiments small movements of the animals disturbed the values of ($V^b - V^m$), but invariably a suitable return was attained within several minutes. The ionic (sodium, potassium and chloride) concentrations in the blood were always measured on the conclusion of these experiments because a knowledge of ΔV_i for the individual ions i was required before any attempt could be made to establish active ion transport in certain physiological conditions.

4.42 Experiments on Blennies

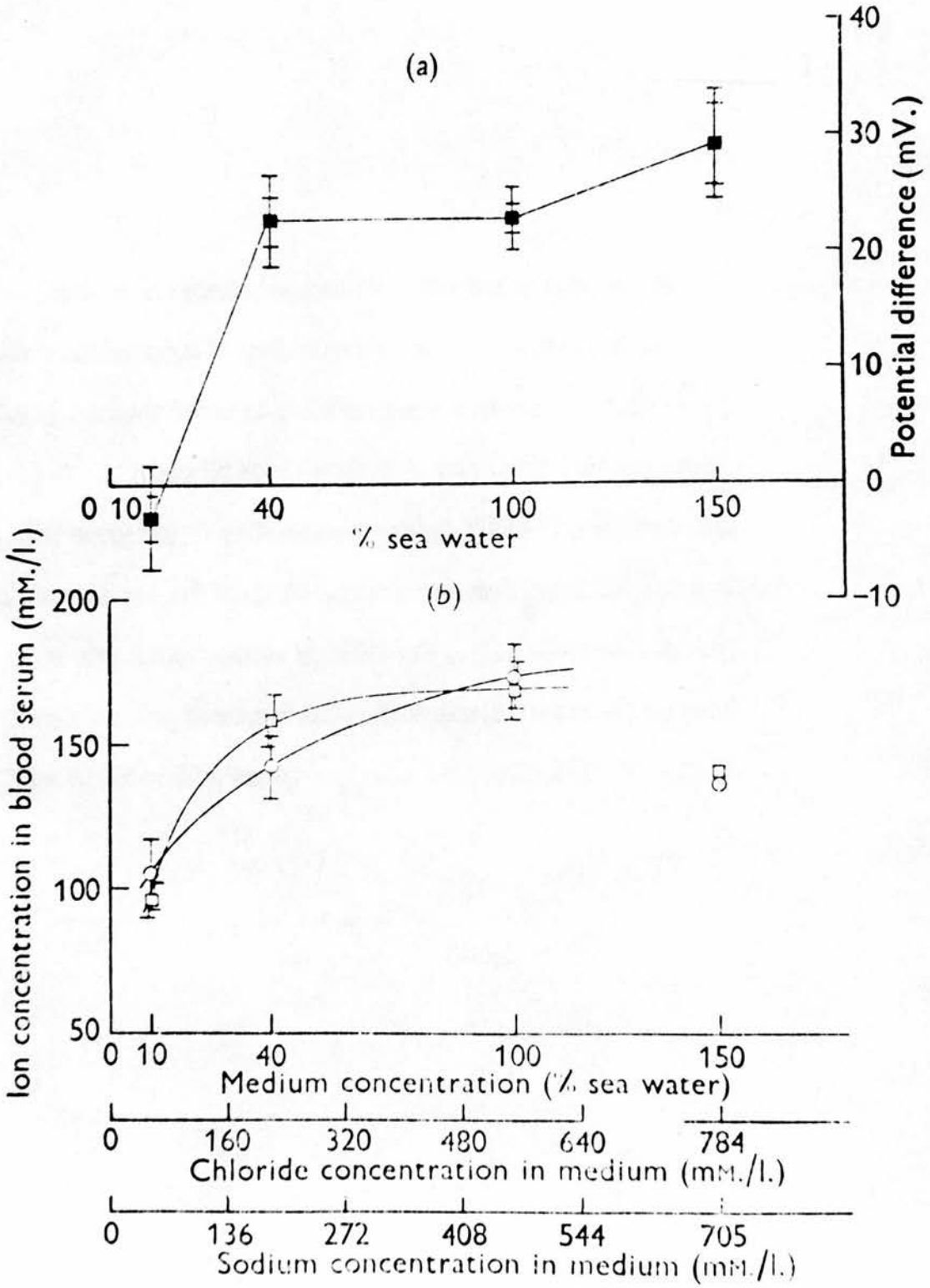
The results of the determinations of the sodium and chloride concentrations in the blood serum and of the potential measurements are shown in Fig. 3. The general type of variation of ($V^b - V^m$) with time is shown in Fig. 2 for two fish in different media.

4.43 Experiments on Isolated Intestine of Cottus

Respiration measurements in a Warburg apparatus gave the mean $\pm$ SD rate of oxygen consumption of six pieces of intestine (3 animals) over a total of 35 hours in Ringer as 0.4 ± 0.2 μl. Oxygen/dry weight (mg). hr at 25°C. Since a piece of intestine of unit area (1 cm^2) had a dry weight of about 10 mg, the oxygen consumption of isolated intestine can be expressed as 4 $\mu l.cm^{-2}hr^{-1}$.

Observations of the net water flux (mucosa to serosa) across 22 pieces of small intestine from 16 fish gave a mean $\pm$ SE of 4.9 ± 0.5 mg $cm^{-2}hr^{-1}$ (35 measurements). In four pieces of tissue exhibiting a net transport of water the movement

Fig. 3. (a) The relation of potential difference between blood and medium in animals, adapted to various concentrations of external medium. (b) Sodium and chloride concentrations in blood serum. In (a) the mean potential difference ■ is shown with SE and SD. In (b) sodium concentration □ and chloride concentration ○ are given with SE. It should be noted that, for concentrations of the external medium greater than 100% S.W., the chloride concentration axis is not linear because the sodium concentration was increased proportionately to give 150% S.W. by adding a suitable quantity of sodium chloride.


(a)
40
30
20
10
0
−10
Potential difference (mV.)
0 10
40
100
150
% sea water
(b)
200
150
100
50
Ion concentration in blood serum (mM./l.)
0 10
40
100
150
Medium concentration (% sea water)
0
160
320
480
640
784
Chloride concentration in medium (mM./l.)
0
136
272
408
544
705
Sodium concentration in medium (mM./l.)

was halted by the addition of potassium cyanide to the serosal medium (final concentration of CN^-, 1 mM/l.).

Measurements of the potential difference existing across 9 pieces of small intestine, bathed on both sides by Ringer, gave 0.0 ± 0.2 mV (mean $\pm$ SE). Since there exists no measurable potential difference across this epithelium the magnitudes of the fluxes of any ion in both directions determine whether there is active transport of the ion or not.

Values of mucosal to serosal sodium flux, ${}_mJ_s$, and of serosal to mucosal sodium flux, ${}_sJ_m$, in the same sacs are shown in Table 2.

TABLE 2

SODIUM FLUXES ACROSS ISOLATED INTESTINE

Sac No.	Sodium Fluxes (μ.equiv.$cm^{-2}hr^{-1}$)		
	${}_sJ_m$	${}_mJ_s$	${}_mJ_s^{net}$
1	13	30	17
2	14	26	12
3	15	21	6
4	12	23	10
5	12	25	13
6	13	23	10
7	19	34	15
8	9	19	10
9	15	25	10
Mean $\pm$ SE	14 ± 1	28 ± 2	12 ± 1

was halted by the addition of potassium cyanide to the serosal medium (final concentration of CN^-, 1 mM/l.).

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5	12	25	13
6	13	23	10
7	19	34	15
8	9	19	10
9	15	25	10
Mean $\pm$ SE	14 ± 1	28 ± 2	12 ± 1

4.5 DISCUSSION

4.51 Ionic Regulation in Goldfish

In performing the in vivo potential measurements it was assumed that the extracellular fluid of the animal was in flux equilibrium with the external medium i.e. the concentration of every ion in the blood serum was maintained at a steady value.

If an ion is moving passively under the action of the physical forces produced by chemical and electrical potential gradients then $(V^b - V^m)$ will be equal in magnitude and sign to the Nernst potential for the ion. In artificial pond water it appears that both sodium and chloride ions are being 'pumped' into the animal. This active transport presumably takes place at the gills to balance ionic loss in the urine flow. Support for this view exists in the calculations of the ratio (J_{in}/J_{out}) for sodium and chloride ions from equation (6); this calculation assumes that the potential difference across the gill epithelium is equal to $(V^b - V^m)$ and gives a ratio of about 0.05 for both ions. Clearly, purely passive flows of these ions cannot satisfy conditions of ionic regulation in this species.

The role of potassium ions is difficult to assess because of the inaccuracy in the measurements of serum concentration of this ion.

The evidence presented here for active absorption of ions agrees well with the conclusions of other workers (Meyer, Krogh et al.) from flux measurements on this animal. Until this problem is attacked at the level of in vitro studies on isolated gills, however, no unequivocal proof of active ion transport in goldfish can be claimed.

4.52 Ionic Regulation in the Blenny

Table 3 gives the values of the Nernst potentials for sodium and chloride along with the mean ± SE value of $(V^b - V^m)$ for blennies adapted to various

external media. Applying the same criteria as above, it is difficult to decide whether sodium is moving passively or being actively 'pumped' out from animals in 100 and 150% S.W. In 10 and 40% S.W. it appears that sodium is not in passive equilibrium and the data suggest an active absorption. It also appears from Table 3 that chloride is not in passive equilibrium in all of the media.

TABLE 3

SODIUM AND CHLORIDE NERNST POTENTIALS AND $(V^b - V^m)$

Medium (% S.W.)	ΔV_{Na} Mean ± SE (mV.)	ΔV_{Cl} Mean ± SE (mV.)	$(V^b - V^m)$ Mean ± SE (mV.)
10	- 18 ± 3	+ 16 ± 3	- 3 ± 2
40	+ 4 ± 2	- 11 ± 2	+23 ± 2
100	+ 26 ± 2	- 29 ± 2	+23 ± 1
150	+36 ± 2	- 38 ± 2	+ 30 ± 4

The most straightforward explanation of these data is that in the hyper-osmotic media (i.e. 40, 100 and 150 % S.W.) the chloride ions are actively excreted by the gills, while in 10% S.W. the chloride 'pump' changes direction and there is also an active influx of sodium in 10 and 40% S.W.

Again the nature of potassium movements is difficult to describe on this basis. In all media the serum concentrations were found to be larger than 10 mM/l. whereas the data given by Lockwood (1961) on the blood of marine teleosts show values between 2 and 8 mM/l. for potassium. If a more likely (constant) value of 5 mM/l. is assumed, this would suggest that potassium ions might be in passive equilibrium in 100% S.W. and actively absorbed from 10 and 40% S.W.

Although the potential measurements in 100% S.W. were inconclusive in determining the nature of sodium transport in the blenny, House (1963) concluded from

flux measurements that animals, adapted to 100% S.W., actively excreted sodium ions.

Calculations of (J_{in}/J_{out}) from equation (6) for sodium and chloride ions (cf. 4.51 in 100 and 10% S.W. also suggest that across the gills there is an active efflux of chloride from animals in 100% S.W. and that there are active sodium and chloride influxes in animals in 10% S.W.

4.53 The Isolated Intestine of Cottus

The measurements of the net flow of water across the isolated intestine gave an average net flux of 5 mg $cm^{-2}hr^{-1}$ into the serosal medium and this water transport was ultimately dependent on the metabolism of the tissue. These observations confirm the view of Homer Smith and others that there is a net uptake of water in the intestine of marine teleosts and that this flow is dependent, at least indirectly, on the tissue metabolism. This result is also in good agreement with numerous studies on the intestine of other vertebrates since net absorption of water of about the same magnitude (ca. 10 mg $cm^{-2}hr^{-1}$) occurs in all of these tissues.

It is clear from Table 2 that there is active sodium transport from the mucosal to serosal surfaces of Cottus intestine. However, as the potential difference across this tissue was zero, I suspect that there must be either net anion transport in the direction of active sodium flow or net cation transport in the reverse direction. The interesting question of whether there is active anion transport or not remains to be investigated. These observations tend to support previous measurements on ion absorption by marine teleosts as it appears that sodium and perhaps chloride ions are actively absorbed by the intestine.

As the oxygen consumption of the isolated intestine has been measured it is possible to calculate what fraction of metabolic energy is employed in the absorption

processes. Assuming that water absorption in the intestine is a passive concomitant of the active sodium flux, then the only work done is that required to overcome the internal sodium resistance of the skin, viz. RT ln $({}_mJ_s/{}_sJ_m)$. Therefore, the work done in transporting 10 μ equivalents $({}_mJ_s^{net})$ across 1 cm^2 per hour at room temperature is about 4×10^{-3} calories, whereas the oxygen consumption rate of the tissue is 4 $\mu l.cm^{-2}hr^{-1}$. On this basis the total apparent amount of metabolic energy available for sodium transport is about 18×10^{-3} cal. $cm^{-2}hr^{-1}$, and the active sodium transport is therefore within the energetic limits set by metabolism. However, this calculation takes no account of the possible work done in the transport of water or other ions.

The nature of the water transport across the intestine remains to be studied, but prima facie there seems to exist a significant difference in kinetic mechanism compared to those proposed in other intestinal epithelia. The ratio of the number of water molecules transported per sodium ion transported is considerably lower than, for example, that of the rat ileum (Curran, 1960) i.e. 30 compared to 300. This ratio is probably not a reliable criterion for the linkage-mechanism of water and solute flows, but this low value might indicate a different kind of coupling, if any, between these fluxes in Cottus intestine from that usually found in the intestine of vertebrates.

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